Accelerating antischistosomal drug discovery: Preclinical studies of antimalarials, synthetic peroxides and praziquantel derivatives

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Summary

Schistosomiasis is one of the most devastating parasitic diseases in tropical countries and remains a major public health problem, especially in Sub-Saharan Africa. It is caused by blood flukes of the species *Schistosoma*. *Schistosoma haematobium*, *S. mansoni* and *S. japonicum* are the main species responsible for the largest number of cases. Chronic schistosomiasis is caused by the immunological response to eggs trapped in tissue and organs. In the case of *S. mansoni,* eggs get trapped in the gut wall and liver, leading to severe tissue destruction. Treatment with praziquantel (PZQ) is the mainstay of morbidity control. PZQ is safe and efficacious, but it is the only available drug. Moreover, it has a major efficacy limitation due to lacking efficacy on juvenile *Schistosoma* stages. Hence new backup drugs are strongly required. Antischistosomal drug discovery has been neglected for a long time. As a consequence, the antischistosomal drug pipeline is empty. Technical innovations for antischistosomal drug screening and new promising drug candidates are urgently needed.

My PhD thesis had two major aims. The first was to improve the drug screening process by evaluating new assays and readouts and by refining the screening cascade. The second was to further investigate known lead compounds and to identify entirely new drug candidates and chemical scaffolds.

A literature review conducted at the beginning of my PhD thesis revealed that clinical trials conducted in pediatrics, are mainly concerned with antimalarials and only 3 % are dealing with schistosomiasis. Especially the field of PK trials is completely neglected and the importance of investigating the relationship of infections and PK changes was emphasized.

The common blood-feeding characteristic of schistosomes and *Plasmodia* has led to studies with antimalarial drugs against schistosomes in recent years. Amongst different chemical structures, mefloquine (MFQ), a 4-quinolinemethanol, and the artemisinins, with their distinct peroxidic scaffold, qualified as leads for antischistosomal drug discovery.

The in vitro and in vivo antischistosomal potential of selected MFQ-related arylmethanols was characterized. Furthermore the role of various iron sources in in vitro drug activity was investigated to get insights into the mode of action (MOA). Pharmacokinetic (PK) and pharmacodynamic (PD) properties of lead candidates were explored in *S. mansoni* infected and uninfected mice.

The class of 4-quinolinemethanols revealed the best in vitro activity (IC_{50}s $\leq$ 3.5 μM) against adult schistosomes. A ten-fold increase in activity was observed for the two lead 4-quinolinemethanols (MFQ, WR7930) when incubated in the presence of hemoglobin. High worm burden reductions (83 - 100%) were observed for EP (4-pyridinemethanol) and WR7930 (4-quinolinemethanol) in mice harboring adult *S. mansoni* or *S. haematobium*. EP and MFQ were selected for further PK investigations. A HPLC-UV method was successfully validated to measure the two drugs of interest simultaneously within mouse plasma. The migration of schistosomes to the liver after treatment with active drugs, known as hepatic shift, was delayed for both drugs (72 - 168 h). Dramatic changes in the drug disposition of MFQ and EP were triggered by the *S. mansoni* infection. Increased AUCs and half-lives led to slowed drug clearance.

Driven by these promising antischistosomal properties of antimalarials the MMV Box, containing 200 drug-like and 200 probe-like compounds, was investigated. Two entirely new chemical scaffolds, the diarylureas and the dianilinoquinoxalines, presented excellent in vitro activity (IC_{50}: 0.8 μM) as well as moderate in vivo worm burden reductions (WBR: 40.8 - 52.5 %) following single oral drug administration in *S. mansoni* infected mice.

To pursue the active peroxidic scaffold further, various promising peroxide classes- ozonides (OZs), 3-alkoxy-1, 2-dioxolanes, tetraoxanes, tricyclic monoperoxides and alphaperoxides- were tested against juvenile and adult *S. mansoni* stages. Additionally the roles of iron and the peroxidic core in drug activity were evaluated.

Promising in vitro activity on both stages was observed for the alkoxydioxolanes. However, only moderate, non-significant activity was observed in vivo. Iron sources did not alter activity on schistosomes, supporting an iron-independent MOA. Non-peroxidic alkoxydioxolane analogues lacked activity against both parasites, underlining the necessity of a peroxide functional group.

Investigations on the three new peroxidic classes (tetraoxanes, tricyclic monoperoxides and alphaperoxides) aimed to get more insights on the structural needs of peroxidic drugs for antischistosomal activity. High in vitro activitiy was revealed on the schistosomular stage, but decreased susceptibilities were observed on the adult stage. One tetraoxane and one tricyclic monoperoxide presented good in vivo activity against adult *Schistosoma* infections in mice but lacked efficacy on the juvenile infection in vivo. A non-iron dependent activation was observed for these classes as well.

Both lead candidates represent new chemical scaffolds but come along with cytotoxicity limitations. Furthermore both compounds are at the very early stage of drug development, and extensive further research is needed for successful drug development. The most promising peroxidic lead candidate was elucidated amongst the OZs, OZ418, with excellent in vivo activity in both juvenile and adult *Schistosoma* infections.

Additionally we were interested in the antischistosomal activity of PZQ derivatives resulting from novel organometallic derivatization strategies. However organometallic ferrocenyl PZQ derivatives presented only moderate in vitro effects against adult worms. The derivatization of PZQ with chromium moieties led to promising in vitro results but has so far shown to be inefficacious in vivo.

During the process of evaluating this broad range of chemical scaffolds and lead candidates we worked on the improvement of the screening cascade, identifying new parameters resulting in improved in vitro - in vivo correlation, evaluating cut-offs and their impact on hit decision as well as investigating new readout tools for drug screenings assays. During the work of this thesis an in vitro motility assay based on *S. haematobium* schistosomula was successfully developed. Colorimetric markers were evaluated as an alternative readout technique, but no promising results were achieved so far.

In conclusion, MFQ and EP are interesting antischistosomal lead candidates, however their extensive half-lives and slow clearance might be an issue regarding the strict safety profile. In general antimalarials presented an excellent starting point for antischistosomal drug discovery. Amongst the peroxidic classes, OZ418 showed great potential for a good lead candidate.

Screening the MMV Box elucidated additionally two entirely novel chemical scaffolds. The PK trials emphasized strong impacts of an adult *Schistosoma* infection on PK parameters and underlined the need for further studies in that field. Finally interesting parameters for the antischistosomal drug-screening cascade were elucidated in the course of this thesis.

Table of Abbreviations

ADME	Absorption, Distribution, Metabolism, Elimination
AUC	Area under the plasma concentration time curve
C_{max}	Maximal plasma concentration
CQ	Chloroquine
CV	Coefficient of variation
DnDI	Drugs for Neglected Diseases initiative
EP	Enpiroline
FQ	Ferroquine
FWBR	Female worm burden reduction
HPLC	High pressure liquid chromatography
HTS	High-Throughput Screening
$IC_{50/90}$	Concentration required to kill 50/90 % of the parasites
iFCS	Inactivated fetal calf serum
IS	Internal standard
KW	Kruskal-Wallis test
LLOQ	Lower limit of quantification
MFQ	Mefloquine
MMV	Medicines for Malaria Venture
MOA	Mode of action
MW	Molecular weight

NTDs	Neglected tropical diseases
NTS	Newly Transformed Schistosomula
OZ	Ozonide, Synthetic peroxide, 1,2,4-trioxolane
PCR	Polymerase chain reaction
PD	Pharmacodynamic
PK	Pharmacokinetic
PPP	Public Private Partnership
PZQ	Praziquantel
QC	Quality control
RRE	Relative recovery
RSD	Relative standard deviation
SAR	Structure-activity relationships
SD	Standard deviation
SE	Standard error
$t_{1/2}$	Elimination half-life
T_{max}	Time to achieve maximal plasma concentration
ULOQ	Upper limit of quantification
USFDA	US Food and Drug Administration
UV	Ultra-violet light
WBR	Worm burden reduction

Chapter 1

General Introduction

1 Schistosomiasis

1.1 Epidemiology

Schistosomiasis is an infectious disease common in the tropics and sub-tropics, and is caused by parasitic blood-dwelling worms of the genus *Schistosoma* (Gryseels et al., 2006; Molyneux et al., 2005). It belongs to the so-called neglected tropical diseases (NTDs) and affects primarily poor people in rural settings of the developing world (WHO, 2012, 2013b). There are two major forms of schistosomiasis – hepatic and urogenital – caused by five different species of blood flukes. Hepatic and intestinal schistosomiasis is mainly caused by *Schistosoma mansoni* (occurring in Africa, South America so as the Middle East) and *S. japonicum* (China, Indonesia and the Philippines). The urogenital form is exclusively caused by *S. haematobium* (Africa, the Middle East) (Gryseels et al., 2006; WHO, 2013b). Two further human species are *S. mekongi* (Laos and Cambodia) and *S. intercalatum* (rainforest areas of central Africa), which are more geographically localized (Figure 1) (CDC, 2013).

According to last figures from 2003, more than 10 % of the worlds' population, 779 million people, lives at risk for infections with schistosomes and an estimated 207 million people are infected with at least one of the *Schistosoma* species (Steinmann et al., 2006). Most schistosomiasis cases worldwide are caused by *S. haematobium* (approx. 64 %) and *S. mansoni* (approx. 34%) (Hotez et al., 2006).

Based on the latest estimates of the burden of disease, schistosomiasis causes the loss of 3.3 million DALYs. To note, numbers have slightly increased compared to estimates 20 years ago and could have a great uncertainty range (Murray et al., 2012).

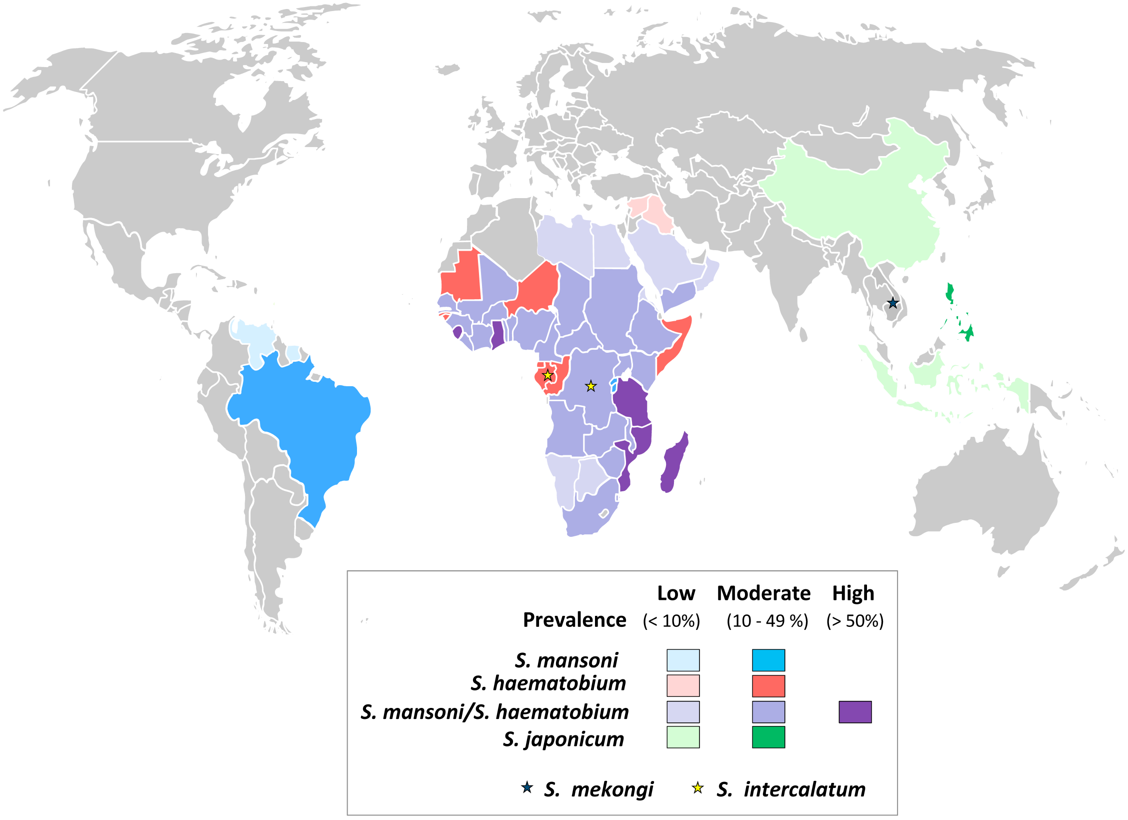


Figure 1: Distribution of the five human *Schistosoma* species (*S. mansoni, S. haematobium*, *S. japonicum*, *S. intercalatum* and *S. mekongi*). The prevalence for each country worldwide is indicated by the color code. Adapted from (Gryseels et al., 2006) and (WHO, 2013b).

1.2 Biology and Lifecycle

Schistosomiasis belongs to the water-borne diseases. Infections take place following contact with water that is contaminated with the infectious larval stage (cercariae) (Figure 2). Typical sources of infection are natural streams, ponds and lakes. However, environmental changes resulting in the development of water resources, and the growth and migration of populations, can facilitate the spread of schistosomiasis (Ross et al., 2002). Cercariae enter the human host via skin penetration within minutes (Haas and Haeberlein, 2009). By penetrating the skin of the human host, cercariae loose their forked tail and convert to schistosomula (Figure 3B). The schistosomular stage migrates to the lungs, where they transform to juvenile worms. From there, worms continue their migration via the venous system to the heart, and finally reach the liver where they mature (within 4-6 weeks post infection) to adult male and female worms (Gryseels et al., 2006). Adult schistosomes are 1-2 cm in length and have separate sexes. The thinner and longer female lives within the gynaecophoric channel formed by the male’s body (Figure 3 C) (Gryseels et al., 2006). From the liver and through the vasculature, they reach their final destinations where they reside. The final destinations are species dependent: the paired (male and female) *S. mansoni*, *S. japonicum*, *S. intercalatum* and *S. mekongi* worms migrate to the mesenteric venous plexus and portal vein, whereas *S. haematobium* species reside in the venous system around the bladder (perivesical venous plexus). Schistosomes feed on host blood and plasma proteins via anaerobic glycolysis. Females start laying hundreds to thousands of eggs per day (Figure 3A) (Gryseels et al., 2006). The eggs, containing ciliated miracidiae, migrate back into the lumen of the intestine or bladder by secreting proteolytic enzymes. Eggs that get trapped within the tissue during migration cause an ongoing host immune response resulting in pathological tissue changes (Pearce and MacDonald, 2002). Eggs reaching the lumen of the intestines or bladder get excreted via feces or urine and viable miracidiae hatch upon water contact, light and chemical stimuli. Miracidiae penetrate the intermediate snail host (*S. mansoni*/ *Biomphalaria* species; *S. haematobium*/ *Bulinus* species; *S. japonicum*/ *Oncomelania* species). Within the snail the miracidiae go through two generations of sporocysts to develop into cercariae, which finally get released starting the lifecycle all over again (Figure 2)(Gryseels et al., 2006). For this work, the two major *Schistosoma* species of interest are *S. mansoni* and *S. haematobium*.

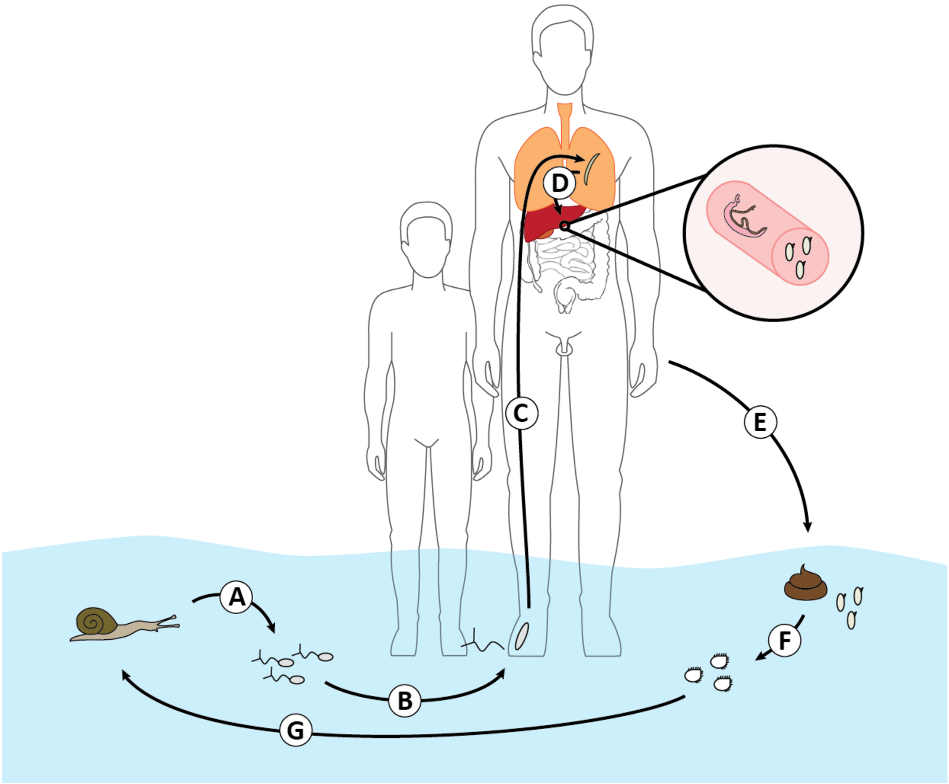


Figure 2: The lifecycle of *S. mansoni*: (**A**) Infected snails shed cercariae, which penetrate the skin of the human host, (**B**) loose their forked tail and transform to schistosomula. (**C**) They migrate to the lungs and develop into juvenile worms. (**D**) From there juveniles migrate to the liver, mature and start laying eggs within the blood vessels. (**E**) Eggs migrate back into the gut lumen and get excreted via feces. (**F**) Following water contact miracidiae hatch from vital eggs and (**G**) infect the intermediate host snails.

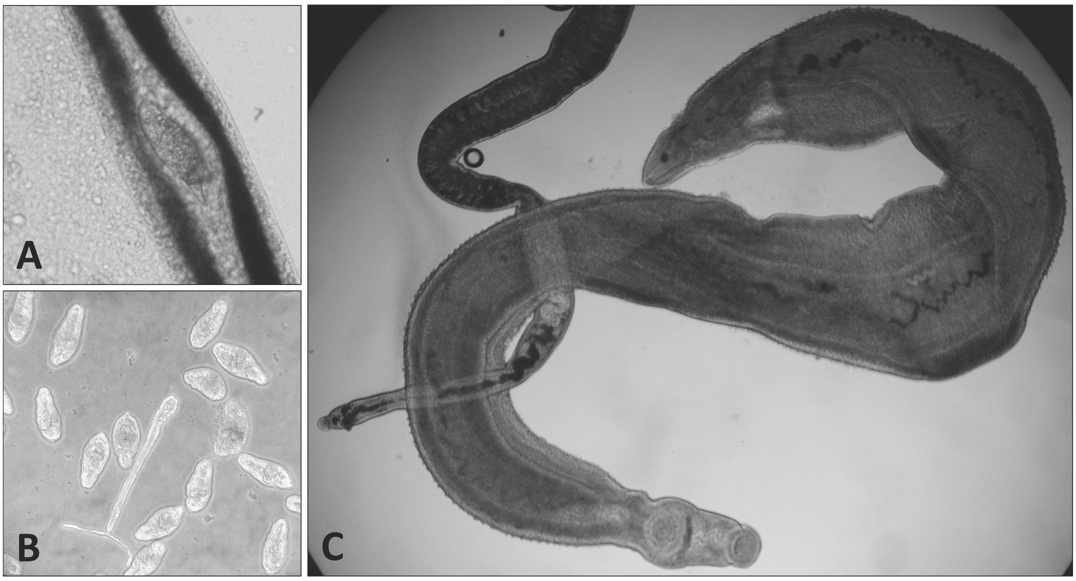


Figure 3: (**A**) Egg within the gut of a female *S. mansoni,* lateral spine visible. (**B**) Newly transformed schistosomula (NTS) and a forked-tail, lost during mechanical transformation of cercariae. (**C**) Adult *S. mansoni* worms, a female (thin) lays in the gynaecophoral channel of a male schistosome. The black lines visible within guts represent hemozoin, a degradation product of digested host hemoglobin.

1.3 Pathology and Clinical disease

Schistosomiasis develops into two different courses of disease. Within a few weeks or months after primary infection, acute schistosomiasis occurs. Chronic schistosomiasis develops mainly in people experiencing longstanding infections in poor rural settings (Gryseels et al., 2006).

1.3.1 Acute schistosomiasis

Initially urticarial rash (swimmers' itch) can occur as an early symptom, caused by the skin penetration of cercariae (Bottieau et al., 2006). Acute schistosomiasis, also known as Katayama syndrome, is a systemic hypersensitivity reaction against schistosomula migration and egg deposition (Ross et al., 2007). The acute process is mostly observed as a feverish syndrome, in travelers after first time infection or in individuals exposed to heavy reinfection (Gryseels et al., 2006; Lambertucci et al., 2013; Ross et al., 2007). The clinical manifestations of the acute stages are species-independent (Barsoum et al., 2013). Clinical symptoms are rather non-specific and likely to be misdiagnosed. They involve fever, myalgia, fatigue and respiratory symptoms (such as non-productive cough, infiltrates in chest radiography) in the earlier phase and abdominal symptoms in the later phase. This symptom chronology matches with the development of schistosomes within the human host, from the lung stage to the liver stage (Bottieau et al., 2006; Gryseels et al., 2006). To note, acute symptoms are rarely observed in chronically exposed populations with *S. mansoni* and *S. haematobium*, which might be due to lack of diagnosis (Gryseels et al., 2006). However the Katayama syndrome does occur in previously infected *S. japonicum* cases, and is manifested by severe symptoms (McManus et al., 2010).

1.3.2 Chronic Schistosomiasis

Chronic disease is not caused by adult worms but by a host response to accumulating tissue-trapped eggs, which became trapped during the perivesical or peri-intestinal (depending on the species) migration process (Gryseels et al., 2006; Pearce and MacDonald, 2002). The immune response, induced by egg antigens, orchestrates the development of granulomatous lesions, which are composed of collagen fibers and cells, surrounding the egg. As trapped eggs die, granulomas resolve leaving fibrotic plaques (Boros and Warren, 1970; Pearce and MacDonald, 2002). Hence, the severity of clinical outcomes is related to the infection intensity as well as the individual immune response (Gryseels et al., 2006). Organs that are affected in chronic schistosomiasis depend on the causative *Schistosoma* agents. I will concentrate in this introduction on the outcome of *S. mansoni* and *S. haematobium* since the focus of this thesis is on these two species. To note, *S. japonicum*, *S. mekongi* and S. *intercalatum* cause similar symptoms as described for *S. mansoni*.

- **Genitourinary schistosomiasis**

S. haematobium eggs provoke pathological and physiological changes of the vesical and ureteral wall, causing hematuria and dysuria as common early symptoms (Danso-Appiah et al., 2008; Emukah et al., 2012; Gryseels et al., 2006). Hematuria is the first sign of the established disease, appearing two or three months post infection (Gryseels et al., 2006; Ross et al., 2002). However serious sequelae of persistent *S. haematobium* infections include chronic lesions, which evolve into bladder fibrosis and calcification. Consequently, hydronephrosis and hydroureter can develop, which can eventually lead to kidney failure, illustrating a risk factor for squamous bladder cancer (Alibek et al., 2012). In approximately one third of *S. haematobium* infected women, infections causes genital disease (Poggensee and Feldmeier, 2001; Ross et al., 2002).

- **Hepatic schistosomiasis**

Chronic *S. mansoni* infections cause hepatic pathological and physiological changes in infected patients leading to early inflammatory hepatic schistosomiasis and late fibrotic schistosomiasis. The main clinical symptom of granulomatous inflammation is hepatomegaly in children and adolescence (Gryseels et al., 2006; Ross et al., 2002). Fibrotic hepatic schistosomiasis develops years later (Gryseels et al., 2006). Chronic hepatic schistosomiasis leads to liver cirrhosis, portal hypertension, and premature death (Danso-Appiah et al., 2013). Due to liver cell functions chronic schistosomiasis has pharmacokinetic (PK) implications, thus varying drug profiles occur in infected patients (el Guiniady et al., 1994; Wilby et al., 2013). The impact of chronic schistosomiasis on PK drug profiles has been studied closer in the framework of this thesis (see Chapter 3). Furthermore *S. mansoni* infections cause intestinal pathological and physiological changes. Schistosome eggs migrating through the intestinal wall provoke mucosal granulomatous inflammation situated mainly in the large bowel and rectum (Figure 4). This leads to symptoms such as chronic or intermittent abdominal pain and discomfort, loss of appetite, as well as diarrhea and blood in stool (Danso-Appiah et al., 2013; Gryseels et al., 2006).

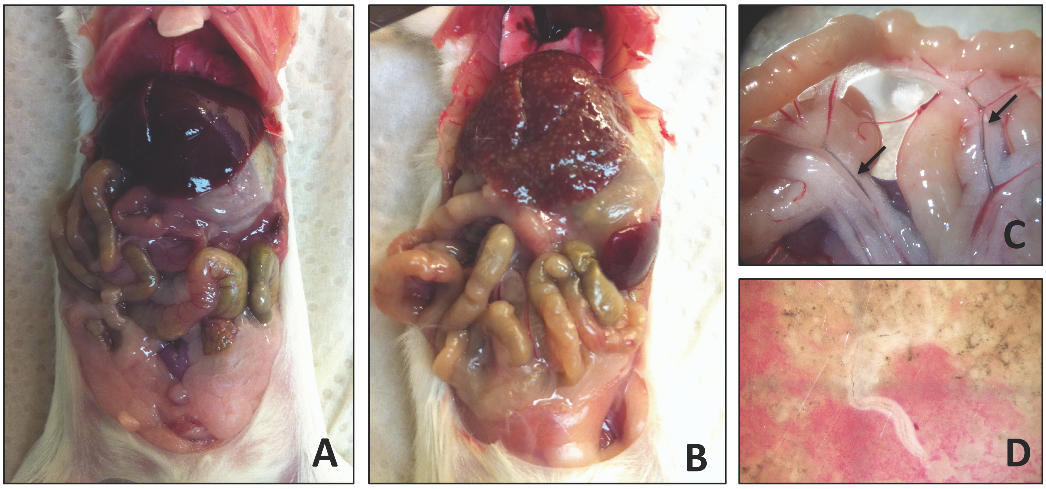


Figure 4: (**A**) Liver and intestine of an uninfected NMRI mouse. (**B**) Chronic infected liver (7 weeks post infection) as well as an inflamed and swollen intestine and an enlarged spleen. (**C**) *Schistosoma* pairs residing in mesenterial veins (arrows point out worms), (**D**) Adult schistosome within a pressed liver.

1.4 Impact of Schistosomiasis on Public Health

Schistosomiasis has been associated with growth retardation, under nutrition, anemia, possible cognitive and memory impairment as well as poor work productivity, which limit the potential of infected populations and reduce the chance to combat rural poverty (Gray et al., 2011; King, 2010; King and Dangerfield-Cha, 2008). Improving maternal health is one of the WHO millennium goals. However, in Africa alone an estimated 10 million pregnant women are infected with schistosomes. Half of theses women consequently develops anemia, which causes complications during pregnancy including low birth rate and increased maternal morbidity. Furthermore schistosomiasis contributes to infertility affecting female reproductive health. Women who have urogenital schistosomiasis are 3-4 times more susceptible to a HIV infection (WHO, 2013b). It is important to keep in mind that infectious diseases such as schistosomiasis contribute to lifelong impairment in already disadvantaged groups. Acquiring schistosomiasis at an early age has life- long detrimental consequences. Therefore sustainable control and intervention strategies are strongly needed to reduce the impact of schistosomiasis on affected people (DCPP, 2008; Hotez et al., 2009).

1.5 Diagnosis

To date, the diagnostic gold standard is the microscopic detection of eggs in stool (*S. mansoni* and *S. japonicum*) or urine (*S. haematobium*) samples. It is easy to identify *Schistosoma* eggs and to define the species, owing to their characteristic size and shape with a lateral (*S. mansoni*) or a terminal (*S. haematobium*) spine (Danso-Appiah et al., 2013; Gray et al., 2011; Gryseels et al., 2006). Diagnosis using direct wet slides is not very sensitive. If no eggs are found, concentration methods, such as formalin-based sedimentation technique, filtration or centrifugation should be utilized to detect low intensity infections (Gryseels et al., 2006). Quantitative methods are recommended for epidemiological purposes and control programs (Bergquist et al., 2009; Danso-Appiah et al., 2013). For intestinal schistosomiasis the Kato-Katz method is commonly used as a quantitative technique (Booth et al., 2003; Katz et al., 1972). Further fecal diagnostic methods include the thick smear and the only recently applied FLOTAC technique for *S. mansoni*, which needs to be investigated further (Glinz et al., 2010; Knopp et al., 2009; Teesdale and Amin, 1976). Egg outputs are influenced by several factors, such as day-to day (Engels et al., 1996), intra-stool and seasonal variation and environmental conditions. Hence single microscopic examinations of stools are unreliable and egg reduction rates should not be over-interpreted as a measure of disease (Danso-Appiah et al., 2013; Enk et al., 2008; Gryseels et al., 2006). Antibody based assays are quite sensitive. A monoclonal antibody-based urine dipstick, which detects circulating cathodic antigen (CCA), is available for diagnosis of the infection with promising results (Coulibaly et al., 2011; Tchuem Tchuente et al., 2012). Another sensitive diagnostic technique, mainly available in European reference laboratories, is the polymerase chain reaction (PCR) based technique (Enk et al., 2012). Further clinical diagnosis options are the ultrasound for pathological changes and the evaluation of clinical symptoms such as hematuria, diarrhea and blood in stool. These options are not very sensitive regarding multiple possible causes in endemic settings (Danso-Appiah et al., 2013; Gryseels et al., 2006). Finally, antibody detection can be useful in a few specific circumstances, such as in patients with Katayama syndrome who are not yet secreting eggs, however its application is limited (Gray et al., 2011).

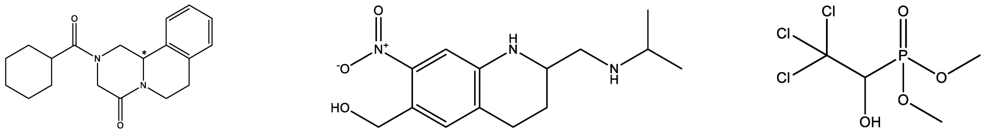
1.6 Intervention and Control

There are three major interventions for the control of schistosomiasis comprising anthelmintic drug treatment, sanitation and health education (Hotez et al., 2006; Rollinson et al., 2012; WHO, 2013b). Chemotherapy is the mainstay of control. It has been specified by the WHO as most rapid and cost-effective intervention, to prevent and reduce morbidity ("preventive chemotherapy") due to schistosomiasis (WHO, 2013a). Since 2001, a WHO resolution (WHA 54.1) officially endorsed preventive chemotherapy as the key public health strategy to combat schistosomiasis. In 2006 the target population was expanded (WHO, 2013a). In 2010, 26 of the 51 countries where large-scale treatment for schistosomiasis was required were implementing treatment programs. Only recently, the WHO set the goal to improve access to treatment, with an estimated 235 million people to be treated by 2018 (WHO, 2013a). Praziquantel (PZQ), which will be discussed in further detail below, is the drug of choice and widely administered in these programs.

The combination of morbidity control with drug treatment and complementary public health interventions is also termed the "integrated control approach" (Knopp et al., 2012; Utzinger et al., 2003). Complementary public health interventions include improving the sanitation and providing safe drinking water, but also mediating hygiene and health education to affected populations (Utzinger et al., 2003; WHO, 2013b). Additionally, vector control has played a major role in controlling and interrupting transmission by utilizing molluscicides (Knopp et al., 2012; WHO, 2013a).

1.7 Chemotherapy

To date PZQ is the drug of choice against all *Schistosoma* species. Since its discovery in the 1970's by Bayer, it has been the main drug used in preventive chemotherapy and will remain the drug of choice in the years to come (Cioli and Pica-Mattoccia, 2003; Utzinger and Keiser, 2004; WHO, 2006). PZQ is used at a dosage of 40 mg/kg and a frequency that is defined based on prevalence (WHO, 2006). PZQ is safe, orally active, rapidly absorbed and shows a rapid onset of action by disrupting the Ca^{++} homeostasis in schistosomes, leading to spastic paralysis caused by rapid Ca^{++} influx (Cioli and Pica-Mattoccia, 2003; Keiser and Utzinger, 2007a; Utzinger and Keiser, 2004). Oxamniquine was discovered in the 1960's by Pfizer and has been used in schistosomiasis control programs against *S. mansoni* mainly in Brazil. The mode of action is well understood: the nucleic acid metabolism is irreversibly inhibited by alkylation. The third antischistosomal drug, which was recommended by the WHO, is metrifonate (Figure 5). This organophorus cholinesterase inhibitor was already introduced in the 1950's and shows good activities against *S. haematobium* after three oral doses (7.5 mg/kg each) (Danso-Appiah et al., 2008). However both drugs have been replaced by PZQ because of its superior clinical, economical and operational profiles (Cioli and Pica-Mattoccia, 2003).



1 2 3

Figure 5: Chemical structures of the three known antischistosomal drugs (**1**) praziquantel, (**2**) oxamniquine and (**3**) metrifonate.

1.8 Need for new drugs

The number of people treated for schistosomiasis with PZQ in preventive chemotherapy programs has almost tripled between 2006 and 2010. However, this number still represents only 13% (35 million people) of the people requiring preventive chemotherapy for schistosomiasis. By 2018 the WHO set the goal of treating an estimated 235 million people (WHO, 2013a). This shift in global health policy towards expanding preventive chemotherapy with only one available drug places new selective pressure on the parasite, which may accelerate the emergence of resistance (Caffrey and Secor, 2011; Fenwick and Webster, 2006). Cure rates following standard single 40 mg/kg oral doses of PZQ treatment already varied substantially across clinical studies (Botros and Bennett, 2007; Danso-Appiah et al., 2013; Gryseels et al., 2001). Reduced susceptibility to PZQ was detected for schistosomes isolated from Egyptian patients who failed to be cured (Ismail et al., 1999). The major weakness of PZQ is its lack of activity against the young developmental *Schistosoma* stage. This might explain treatment failures in areas with high transmission (Botros and Bennett, 2007; Cioli and Pica-Mattoccia, 2003; Keiser and Utzinger, 2007a). A vaccine as an alternative control tool is presently not available (Keiser and Utzinger, 2007a; Siddiqui et al., 2011). New drugs with novel modes of action and activity against all *Schistosoma* stages or drugs that would be eligible for combination chemotherapy are therefore urgently needed (Danso-Appiah et al., 2013; Keiser and Utzinger, 2007a).

1.9 Challenges in compound screening

Drug discovery for schistosomiasis has been limited, even when compared with other worm diseases (e.g. nematodes) for which the animal health sector plays a key-role in drug development (Caffrey and Secor, 2011; Geary TG, 2009). The interest in drug discovery for schistosomiasis has been sidetracked by technological limitations. As fully defined in vitro parasite maintenance systems do not exist, clonal organisms or cell lines are not available and robust reverse genetic tools are missing (targeted gene disruption and transgenic parasites). Therefore the classic target-based rational drug design, the applied standard by the pharmaceutical industry, is not feasible for schistosomes (Caffrey and Secor, 2011). However, new inventions might arise in the future, since only recently genomes and comprehensive transcriptome profiles were investigated for *Schistosoma* species (Caffrey and Secor, 2011). Yet it is important to note, that most FDA approved drugs between 1999 and 2008 and all marketed anthelmintics were discovered by phenotypic screening rather than by target-based approaches (Keiser and Utzinger, 2012).

- **In vitro systems:**

In vitro systems for antischistosomal drug discovery strongly rely and concentrate on phenotypic screenings of newly transformed schistosomula (NTS) and adult worms based on microscopic readouts (Ramirez et al., 2007). To date mainly NTS are used as starting material for in vitro studies. Large numbers can be obtained in a cost effective manner. Their use reduces the need for animals, following the 3Rs (reduce, replace, refine) of animal protection principles (Keiser, 2010). Partially automated phenotypic screenings have been studied to scale up the throughput, aiming for high-throughput systems (HTS) (Abdulla et al., 2009). To quantify phenotypes objectively a variety of assay readouts have been explored, often taking advantage of robotics and automated image acquisition technologies (Paveley et al., 2012). These include real-time monitoring of parasites' motility via electrical impendence (X-Celligence) (Smout et al., 2010), isothermal microcalorimetry to measure heatflow changes (Manneck et al., 2011), fluorescence based motility assays with propidium iodide and fluorescein diacetate stainings (Peak et al., 2010) or fluorescent labeled albumin ingested by the parasites (Holtfreter et al., 2010) as well as colorimetric metabolic markers such as Alamar Blue® (Mansour and Bickle, 2010). However all of these quantitative methods still need further investigations. Nearly all described systems are based on NTS since in vitro cultivation of the adult stage is still not possible. Only recently the first target-based screenings were performed with thioredoxin/glutathione reductase (TGR) and peroxiredoxin (Prx) of *S. mansoni* (Simeonov et al., 2008). In the framework of this thesis we investigated possible in vitro assay alternatives and possibilities to expand the drug screening on *S. haematobium* (Chapter 2).

- **In vivo systems**

S. mansoni is the *Schistosoma* model most widely used. Its life cycle is easy to maintain, with mice as the rodent host under laboratories conditions, and is kept in many institutions worldwide. *S. haematobium* however, is much less studied as it is difficult to grow in rodents and the intermediate host snails are much more sensitive to laboratory conditions (Keiser, 2010). Herewith I will briefly introduce procedures used in our facilities (for more details see Chapters 3-5). Animals are subcutaneously infected with infectious cercariae harvested from infected *Biomphalaria* snails. To study drugs, animals are treated either 21 days post infection (to target the juvenile stage) or 49 days post infection (to target the adult stage). Two weeks post treatment, worms are harvested and the number of schistosomes is compared in treated and untreated animals. Schistosomes are obtained and counted by examining the mesenteric veins and the liver for the presence of schistosomes (Xiao et al., 2007). For in vitro studies on adult schistosomes, worms are grown and harvested as described (Keiser, 2010). New approaches to investigate in vivo drug effects involve in vivo imaging of schistosomes (Krautz-Peterson et al., 2009; Salem et al., 2010).

1.10 Investigational drugs: current antischistosomal drug pipeline

Due to the pressing need for backup drugs, intensified efforts were recently made in the field of antischistosomal drug discovery. In particular academia or academic consortia aimed to fill the empty antischistosomal drug pipeline (Caffrey and Secor, 2011; Keiser, 2010). As described above, antischistosomal drug discovery still relies and concentrates mainly on phenotypic screenings. It is therefore not surprising that only one preclinical lead compound resulted from target-based screenings, namely the oxadiazole-2-oxides. Furoxan, the lead compound, targets the redox enzyme thioredoxin-glutathione reductase (TGR) unique to schistosomes and is currently in preclinical testing (Kuntz et al., 2007; Rai et al., 2009; Sayed et al., 2008; Simeonov et al., 2008). A general attractive starting option for drug development for diseases with limited resources is to work with existing drugs termed repositioning or repurposing of drugs (Caffrey and Secor, 2011; Dissous and Grevelding, 2011). This strategy led to studies with marketed antimalarials against *S. mansoni* (Keiser et al., 2009; Keiser and Utzinger, 2007b, 2012). Worms of the *Schistosoma* species and *Plasmodia*, the parasite causing malaria, are both blood-feeding parasites. The two species present similarities in the digestion of hemoglobin. Both diseases, malaria and schistosomiasis, are often co-endemic, especially in Africa. Therefore, finding an antimalarial that is also effective against schistosomiasis would be optimal in co-endemic regions (Caffrey and Secor, 2011).

Mefloquine (MFQ) evolved as a strong lead candidate with in vitro and in vivo activities against juvenile and mature worms of major *Schistosoma* species (Keiser et al., 2009; Xiao et al., 2009). Recently, its efficacy was proven in clinical trials (Keiser et al., 2010a). The mode of action (MOA) of MFQ is not fully elucidated yet. However several studies noted extensive morphological disturbances on adult schistosomes (Keiser et al., 2009; Manneck et al., 2010). In the field of malaria research it has been suggested that the drug interferes with the hemoglobin digestion, leading to the formation of toxic heme-drug complexes within the malaria parasites (Egan, 2008). Schistosomes also produce hemozoin as a hemoglobin degradation product. It has been shown that quinolinemethanols interfere with the hemozoin formation within

S. mansoni and that inhibition of heme aggregation by chloroquine (CQ) reduces *S. mansoni* infections in mice (Correa Soares et al., 2009; Oliveira et al., 2004; Oliveira et al., 2000). Recently the inhibition of enolase, important for glycolysis, was detected for MFQ in the non hematophagous schistosomula stage (Manneck et al., 2012).

The antischistosomal activities of the antimalarial artemisinin derivatives were extensively studied in vitro, in vivo and in several clinical trials within the last years (Keiser and Utzinger, 2007b, 2012; Sissoko et al., 2009; Utzinger et al., 2001; Xiao and Catto, 1989; Xiao et al., 2004). Artesunate and artemether show activity against schistosomula and juvenile worms, presenting an interesting candidate for combination chemotherapy with PZQ (Utzinger et al., 2002; Utzinger et al., 2000). To note, the superior efficacy of the combination remains uncertain and has to be investigated further (Danso-Appiah et al., 2013). The essential 1,2,4-trioxane core of the artemisinins offers a valuable starting chemical scaffold for modifications. Various biopharmaceutical shortcomings of the semi-synthetic artemisinins such as short half-life, low bioavailability and the dependency on natural raw material, led to the investigation of synthetic trioxolanes (OZs) as antimalarials (Vennerstrom et al., 2004). Several sets of synthetic peroxides were also studied against schistosomes and a potent lead candidate was identified amongst the ozonides (1,2,4-trioxolanes) (Keiser and Utzinger, 2007b, 2012; Xiao et al., 2007).

The MOA of the peroxides against *Plasmodium* is still being debated. Inhibition of the calcium ATPase (PfATP6) and interference with the digestion of hemoglobin have been proposed (Keiser and Utzinger, 2012). The endoperoxide, the bridge within the 1,2,4-trioxane, might undergo reductive cleavage by heme to generate free carbon-centered radicals or carbocations, which then alkylate parasite biomolecules (Keiser and Utzinger, 2007b). However, a metal-independent activation has also been suggested (Keiser and Utzinger, 2012). The relationship between the peroxidic pharmacophor and antischistosomal activity is largely unknown. Enhanced activity of artemether was observed when incubated with additional hemin, supporting an iron-dependent activation (Xiao et al., 2001). However, considering the only moderate activity against adult worms and the good activity against non-blood feeding juveniles, iron-independent mechanisms might be involved (Keiser and Utzinger, 2012). Trioxaquines (lead candidate: PA1259) represent another chemical class combining the peroxidic core with the aminoquinoline moiety. However no comparable oral antischistosomal effects could be observed in terms of worm burden reduction (Portela et al., 2012). Further reported interesting preclinical candidates present encapsulated tatar emetic (to note, it is relatively toxic) in liposomes (de Melo et al., 2003) and the cysteine protease inhibitor (K11777) (Abdulla et al., 2007). Both candidates showed good worm burden reductions after intra-peritoneal administration but have not yet been tested orally, which is one of the crucial requirements for antischistosomal drug candidates (Keiser and Utzinger, 2007a). A myrrh formulation (Mirazid) showed promising efficacies in clinical trials (Abo-Madyan et al., 2004; Sheir et al., 2001; Soliman et al., 2004), but the result could not be confirmed in further clinical trials (Barakat et al., 2005; Keiser and Utzinger, 2007a). The antileishmanial drug miltefosine has been studied in *S. mansoni* infected animals and yielded good worm burden reductions after multiple oral dosing. However shortening the treatment time from 5 to 3 day resulted in a lack of efficacy (Eissa et al., 2011). Studies with PZQ derivatives trying to overcome the PZQ's lack of activity on the juvenile stage have not yet succeeded (Dong et al., 2010; Ronketti et al., 2007).

Table 1: Worm burden reductions (WBR) on different *S. mansoni* stages after oral administration of investigational compounds to mice. (⦿ drugs which were applied intra-peritoneal) **Red**: lacks activity (WBR <40 %); **Blue**: moderate activity (WBR: 40-80%); **Green**: good activity (WBR>80%), **Grey**: data not available. Data obtained from: (Abdulla et al., 2007; Botros et al., 2004; de Melo et al., 2003; Eissa et al., 2011; Keiser et al., 2009; Portela et al., 2012; Sayed et al., 2008; Utzinger et al., 2002; Xiao et al., 2007)

Compound (Year introduced)	**Dosage**	**Stage specifity of investigational drugs against *S. mansoni* in mice, WBR (%)**		
		Schistosomula 7 - 14 d	Juvenile 21 – 28 d	Adult 35 – 49 d
Praziquantel (1977)	1 x 400 mg/kg	15 %	5 %	95 %
Artesunate (1989)	3 x 150 mg/kg		67 %	24 %
Artemether (1999)	3 x 150 mg/kg		88 %	46 %
Mirazid (2001)	5 x 250 mg/kg			27 %
⦿Tartar emetic (2003)	1 x 27 SB/kg			82 %
⦿K117777 (2007)	14 x 25 mg/kg	88 %		57 %
OZ288 (2007)	1 x 400 mg/kg		95 %	52 %
⦿Furoxan (2008)	5 x 10 mg/kg		88 %	94 %
Mefloquine (2009)	1 x 400 mg/kg	90 %	100 %	100 %
Nilutamide (2010)	1 x 400 mg/kg		36 %	85 %
Miltefosine (2011)	5 x 20 mg/kg	91 %	76 %	95 %
PA1259 (2012)	4 x 50 mg/kg		53 %	40 %

Recently the hydantoin derivative nilutamide, marketed as an antiandrogen, was proven to show interesting antischistosomal activities on the adult stage (Keiser et al., 2010b). Additionally various natural products have been investigated on antischistosomal activity but no potential lead candidate was found so far (Ndjonka et al., 2013).

In summary, several new chemical entities have been studied and investigated for antischistosomal potential in recent years (Table 1). However, few compounds were really promising and therefore thorough lead optimization and SAR studies were conducted. Hence, to date there is no antischistosomal drug candidate in drug development (Phase I, II and III) (Stefanakis et al., 2012).

1.11 Aim and objectives

Novel drugs for the treatment of schistosomiasis are urgently needed. There is no vaccine in sight yet and the current mainstay of morbidity control continues to be preventive chemotherapy with PZQ. Since these programs will be sustained, expanded as well as extended and elimination in certain endemic areas will be targeted, development of resistance to PZQ is a threat. The inactivity of PZQ on juvenile *Schistosoma* stages demonstrates alarming shortcomings of the drug of choice. Furthermore, drug discovery and development for schistosomiasis faces several challenges and limitations, which slow down the research progress. Hence, improving screening tools and screening cascades in general is of high importance. My PhD project aimed to improve antischistosomal drug screening processes, to investigate new chemical classes as well as optimize lead structures, and to elucidate the impact of *Schistosoma* infections on PK parameters of drug candidates.

In order to achieve this aim the following objectives were defined:

1) To improve antischistosomal screening tools by investigating an in vitro drug screening assay using *S. haematobium* schistosomula (Chapter 2)
2) To elucidate antischistosomal lead candidates or novel chemical scaffolds amongst compounds with promising antimalarial activity such as mefloquine-related arylmethanols and compounds within the open access MMV malaria box (Chapter 3)
3) To assess the impact of a chronic *S. mansoni* infection on the oral PK profiles of the most promising arylmethanol candidates (mefloquine and enpiroline) (Chapter 4)
4) To investigate the antischistosomal activity of various peroxidic classes: aryl ozonides, 3-alkoxy-1,2-dioxolanes, bridged 1,2,4,5-tetraoxanes, alphaperoxides, and tricyclic monoperoxides (Chapter 5)
5) To explore the antischistosomal potential of organometallic derivatives of the anthelmintic PZQ (Chapter 6)

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Chapter 2

Development of an in vitro drug screening assay using *Schistosoma haematobium* schistosomula

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Development of an *in vitro* drug screening assay using *Schistosoma haematobium* schistosomula

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Abstract

Background: The development of novel antischistosomal drugs is crucial, as currently no vaccine and only a single drug is available for the treatment of schistosomiasis. Fast and accurate *in vitro* assays are urgently needed to identify new drug candidates and research efforts should include *Schistosoma haematobium*. The aim of the present study was to develop a *S. haematobium* drug sensitivity assay based on newly transformed schistosomula (NTS).

Methods: We first undertook comparative studies on the cercarial emergence rhythms of the intermediate host snails *Biomphalaria glabrata* (*S. mansoni*) and *Bulinus truncatus* (*S. haematobium*). Two transformation methods as well as three purification methods were studied on *S. haematobium* cercariae in order to produce a large number of viable and clean NTS. Known antischistosomal drugs were tested in the established NTS assay *in vitro*. Drug effects were evaluated either microscopically or fluorometrically, using a resazurin based viability marker. Microscopically obtained IC_{50} values were compared with results obtained for *S. mansoni*.

Results: A circadian rhythm existed in both snail species. Infected *B. truncatus* snails shed less cercariae than *B. glabrata* during the testing period. The highest transformation rate (69%) of *S. haematobium* cercariae into NTS was obtained with the vortex transformation (mechanical input) and the highest purification factor was observed using Percoll®. The fluorimetric readout based on resazurin was very precise in detecting dead or/and severely damaged schistosomula.

Conclusions: With the use of viability markers such as resazurin, drug screening assays using *S. haematobium* NTS can be efficiently performed. However, drugs acting on the morphology and motility of *S. haematobium* NTS, such as metrifonate are missed. Drug sensitivity assays with NTS of both species, *S. haematobium* and *S. mansoni*, showed very similar results using known antischistosomal drugs. The *S. mansoni* NTS assay might be more suitable as primary screen in drug discovery efforts, which ultimately aim for a broad-spectrum antischistosomal drug as a larger number of *S. mansoni* NTS can be generated.

Keywords: In vitro, Chemotherapy, Schistosoma haematobium, Newly transformed schistosomula, Alamar Blue®, Cercarial rhythm, Percoll®, *Bulinus truncatus*, *Biomphalaria glabrata*

Background

Schistosomiasis remains one of the most prevalent helminthic infections in the world. Human schistosomiasis is caused by three main species including *Schistosoma haematobium* and *Schistosoma mansoni.* Schistosomes have a complex life cycle with snails serving as the intermediary host between the mammalian hosts. Approximately 800 million people are at risk of schistosomiasis in 74 developing countries, with an estimated 200 million people currently infected. Schistosomiasis is a neglected tropical disease, however, just ranks below malaria in terms of its public health importance [1-4].

Control of schistosomiasis relies on preventive chemotherapy programs targeting the entire at risk population [5]. Up to now, praziquantel has been the drug of choice [6-8]. The drug is safe, has a broad therapeutic profile, and is cheap. However, these advantages have impeded the advances in the development of new drug candidates and vaccines [8,9]. In addition, praziquantel is not a

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perfect drug. It has a poor efficacy against immature worms, leading to a decreased ability of praziquantel to completely clear infections [8,9]. In addition, with the threat of drug resistance that accompanies preventive chemotherapy programs it is imperative to develop a novel antischistosomal drug. This drug should have a broad spectrum of trematocidal activity, similar to praziquantel. Given the known biological differences between *S. mansoni* and *S. haematobium,* drug screening should include testing against *S. haematobium*.

In the past years efforts have been made to improve drug sensitivity *in vitro* assays with *S. mansoni* enabling the testing of a larger number of compounds and improved readouts such as those with Alamar Blue® (a resazurin based viability marker) [10]. However, to our knowledge drug screening assays with *S. haematobium* continue to rely mainly on the adult worm.

The aim of the present work was to develop and validate an *in vitro* drug screening assay for *S. haematobium* newly transformed schistosomula (NTS). In a first step the cercarial emergence rhythms of the intermediate hosts of *S. mansoni* and *S. haematobium*, *Biomphalaria glabrata* and *Bulinus truncatus* were studied. Next, two artificial transformation methods for the production of the schistosomula were compared. In addition, the best purification method and optimal culture conditions were established by testing three different methods and several different media [11-14]. Finally, an assay based on the viability marker resazurin, which is the active component of Alamar Blue® and a redox indicator of enzyme activity, using selected compounds of known antischistosomal activity such as praziquantel, mefloquine, artesunate, metrifonate, and oxamniquine was established and the results compared to a standard motility assay using microscopical readout. Results were compared with drug activity observed microscopically against *S. mansoni* NTS.

Methods

Drugs and media

Praziquantel (Sigma Aldrich), mefloquine (kindly provided by Mepha Pharma AG, Switzerland), artesunate (kindly provided by Mepha Pharma AG, Switzerland), metrifonate (kindly provided by Bayer Animal Health, Germany), and oxamniquine (kindly provided by Q. Bickle, London School of Hygiene and Tropical Medicine) were used as antischistosomal compounds. Drugs were dissolved in dimethylsulphoxide (DMSO) (Sigma Aldrich) to obtain drug stock solutions of 10 mg/ml and then diluted into culture media. A resazurin solution was prepared by dissolving 12.5 mg resazurin (Sigma Aldrich) in 100 ml of 1x PBS. Different media such as Basch Medium 169 (prepared in our laboratories), Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) and Medium 199 (Invitrogen, Carlsbad, CA) were tested and compared to obtain optimal culture conditions for the *S. haematobium* NTS. All test media were supplemented with 5% heat-inactivated fetal calf serum (FCS) based on the culture media protocol of Bash 1981 [12] and 200 U/ml penicillin and 200 μg/ml streptomycin (Sigma Aldrich).

Snails and schistosomes

Bulinus truncatus infected with *S. haematobium* were obtained from BEI Resources, NIAID (NIH: *Schistosoma haematobium* exposed *Bulinus truncatus* subsp. *truncatus*, NR-21965). *S. mansoni* infected *B. glabrata* are maintained at the Swiss Tropical and Public Health Institute in Basel. The snails had been individually infected with either 10 miracidiae per snail (*B. truncatus*) or 8 miracidiae per snail (*B. glabrata*). Snails were kept in tanks with dechlorinated tap water in a humid room simulating a 12 hour day and night cycle. First shedding of cercariae occurred 4–6 weeks post infection. Both snail species were then collected in the morning (Size of snails: 6 – 11 mm, number of collected snails: approximately 70–90 of each species) and placed individually into 24 or 48 well plates (1 ml dechlorinated tap water/well). Each well plate was placed under a direct 2000 lux lamp for at least 5 hours. The cercarial suspension of each *B. glabrata*/*B. truncatus* was collected and used for the mechanical and chemical transformation. For the cercarial emergence rhythm studies, *B. glabrata*/*B. truncatus* were collected (n = 90) in the morning at 8 am and put, as described above, in dechlorinated water filled well plates (1 ml/well) until 3 pm. During this period the cercariae suspension of each snail was collected hourly and counted for each snail under a light microscope. The hourly collected cercariae suspension was replaced with dechlorinated tap water.

Vortex transformation

The vortex transformation was performed based on a slightly adapted protocol of Ramalho-Pinto [11]. Briefly, a cercarial suspension was placed on ice for 30–40 minutes in order to reduce parasite motility. Tubes were then centrifuged for 3 minutes at 3000 rpm. The cercarial pellet was resuspended in 2 ml cold Hanks` Balanced Salt Solution (HBSS) containing 2% amphotericin B (Sigma Aldrich). The suspension was vigorously mixed through a pipette followed by 4 minutes vortexing in order to induce tail shedding.

This step was repeated after an incubation of the mixed tail-schistosomula suspension for 20 minutes at 37°C. The transformation rate was calculated by counting the total number of cercariae in the HBSS suspension before transformation, and placing them in relation to the total number of schistosomula obtained after purification. The transformation rate was calculated for five identically performed mechanical transformations.

Glucose induced transformation

The chemical transformation was carried out as described previously [11]. The cercariae suspension was cooled on ice for 30 minutes to reduce parasite motility. After 2 minutes of centrifuging at 2000 rpm, the cercariae suspension was resuspended in 4 ml of 5% glucose and incubated for 10 minutes in a 30°C waterbath. The tails were removed from the bodies using the ice purification method (see next paragraph). The transformation rate was calculated as described above and was calculated for five identically performed chemical transformations.

Purification and culture of schistosomula

The separation of the bodies from the tails by centrifugation on a 70% Percoll® gradient (polyvinylpyrolidone-coated colloidal silica particles) was based on the method of Lazdins *et al.* [13]. 71.7 ml of Percoll® (Sigma Aldrich, starting density 1.13 g/ml) was diluted directly in 10 ml 1.5 M NaCl and filled up to 100 ml with distilled H_2O to obtain a final working solution. 10 ml of the working Percoll® solution was layered on the bottom of a 50 ml Falcon tube. The gradient was topped by carefully adding 5 ml of the transformed schistosomula suspension. The tube was then centrifuged for 10 minutes at 2000 rpm and 5°C. After centrifugation, 5 ml of the sample was collected from the bottom of the tube after perforation using a heated 16-gauge needle. The collected fraction was then diluted in 7 ml of Medium 199 and centrifuged for another 3 minutes at 3000 rpm. The schistosomula pellet was resuspended in 1 ml of fresh, warm supplemented Medium 199.

Another purification method of the schistosomula was based on a simple and easy swirling technique according to F. Lewis *et al.* [14]. The schistosomula suspension was placed on a Petri dish and sufficient warm Medium 199 added so that the bottom of the Petri dish was completely covered. By swirling the dish gently, all schistosomula accumulated in the center and could be transferred into Falcon tubes. The swirling and collecting step was repeated until schistosomules were no longer present in the center of the dish (approximately 4–5 times).

In addition, the ice method was tested for purification. 7 ml of cold HBSS was added to the schistosomula suspension and cooled on ice for 7 minutes. The supernatant was decanted, and the pellet resuspended again in 7 ml of cold HBSS. This step was repeated three times. The pellet that contained the recovered schistosomula was then resuspended in preheated (37°C) supplemented Medium 199.

After the experiment the number of heads and tails were counted using a sample of 50 μl. The ratio was expressed as purification factor. Every method was performed three times identically and the mean purification factor was calculated.

After successfully transforming cercariae into schistosomula and purifying them, the NTS were incubated for at least 12 hours in supplemented Medium 199 at an atmosphere of 37°C with 5% CO_2 until usage to assure complete transformation [10].

Different media, namely supplemented Basch Medium 169, Dulbecco's Modified Eagle's Medium and Medium 199 were tested and compared to obtain optimal culture conditions for the *S. haematobium* NTS. Viability of NTS (150–200 per media) was assessed based on their morphology and motility using a viability scale ranging from 3 (normal activity, no morphological changes) to 0 (dead), as described previously using increments of 0.5 [15].

In vitro S. haematobium drug assay with microscopical or fluorimetric readout

NTS were obtained by mechanical transformation of *S. haematobium* cercariae as described above. The schistosomula suspension was adjusted to a concentration of 2 NTS per μL with supplemented Medium 199. A 3-fold serial dilution was next performed vertically down a flat bottom 96-well plate, to obtain final drug concentrations of 1.1, 3.3, 10, 30, 90 μg/ml after adding 50 μl of the adjusted NTS suspension yielding a final volume of 250 μl per well [16]. Each drug concentration was tested in duplicate and performed at least three times. Live and dead schistosomula (treated with 70% ethanol) served as a positive and negative control.

For the microscopical readout, assays were evaluated under an inverted bright-field light microscope (Carl Zeiss AG, 8 x 10 magnification) 24, 48 and 72 hours post drug exposure.

NTS were evaluated using the viability scale as described above. IC_{50} values were calculated using CompuSyn software (Version 3.0.1, 2007; ComboSyn, Inc).

Furthermore, drug effects were determined with the help of resazurin, a fluorimetric marker for cell viability [17]. Assays were conducted as described above with the exception that 48 hours post drug exposure 20 μl of the prepared resazurin solution was added to each well. Background fluorescence and absorbance of the drug containing medium were determined for each drug dilution. Wells without drug served as controls. After another 24 hours of incubation, fluorescence development was determined (after a total drug incubation time of 72 hours). Fluorescence was measured using a Spectramax M2 plate reader (Molecular Devices) at 530 nm excitation wavelength and 590 nm emission wavelength.

The IC_{50} of each drug was calculated based on the fluorescence detection using the Softmax Pro Program (Molecular Devices).

In vitro S. mansoni drug assay with microscopical readout

NTS from *S. mansoni* were obtained with the same vortex transformation procedure as mentioned above. The ice purification method was sufficient to separate the bodies from the tails. A lower antibiotic dose (1% penicillin-streptomycin mix) was used for the assays, as lower contamination occurred here. Evaluation was done microscopically using the same viability scale (0–3) as mentioned before.

Statistical analysis

Arithmetic Means and standard deviation were calculated using Microsoft Excel® software for cercarial shedding patterns, transformation and purification factors, fluorescent signals and evaluated IC_{50} values. All values were tested for normality. Student's one-sample *t*-test was used to analyze the statistical significance of differences between mean experimental and control values of the fluorescent values for each NTS drug assay. A P-value of < 0.05 was considered significant.

Results

Cercarial emergence rhythm

The cercarial shedding of the two snail species *B. truncatus* and *B. glabrata* followed a clear circadian rhythm, after a simulated 12:12 hour light dark cycle in the laboratory. There was one shedding peak a day which slightly differed in time and intensity according to the species (Figure 1). The daily emergence pattern peak for *B. glabrata* occurred between 11 am and 1 pm, whereas for *B. truncatus* the peak occurred a little earlier between 10 am and 11.30 am. The total number of cercariae shed was 2.7-fold higher for *S. mansoni* infected *Biomphalaria* snails than for *S. haematobium* infected *Bulinus* snails.

Transformation and purification

The highest transformation rate (mean of 69%) was observed for the vortex transformation. The previously described protocol for the vortex transformation [11] had to be slightly modified, such as increasing the number of pipetting (40x), increasing the vortex time (4 minutes) and using HBSS medium supplemented with 2% amphotericin B in order to achieve a continuously high transformation rate. Since high transformation rates were achieved with supplemented HBSS no other media were tested during the transformation procedure. A transformation rate of only 34% (mean value) was obtained with the chemical transformation. Both transformation methods were easy to perform.

In order to obtain the best method for purifying the NTS from the tails shed after transformation, a purification factor was calculated for three purification methods (Percoll®, ice and swirling method). The best purification factor was observed for Percoll® with 24.4 ± 11.4, followed by the swirling method with 11.7 ± 3.2 and the ice method presenting a mean purification factor of 3 ± 1.7.

Optimal culture conditions for *S. haematobium* schistosomula

All media for *S. haematobium* were supplemented with 200 U/ml penicillin and 200 μg/ml streptomycin. This was the ideal concentration of antibiotics to get rid of bacterial contamination that occurred from *B. truncatus* snail excrement, which could not be eliminated during sedimentation in the first vortex transformation steps. Supplemented Medium 199 turned out to be the most

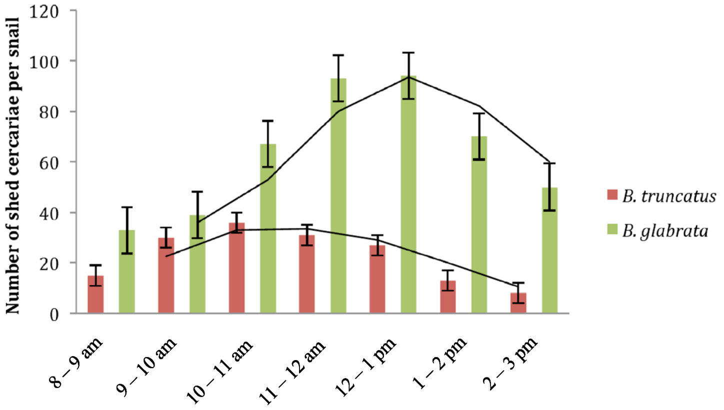

Figure 1 Circadian rhythm of cercariae released from *S. haematobium* infected *B. truncatus* and *S. mansoni* infected *B. glabrata* snails. Mean values of (n = 3) performed experiments are shown, bars indicate standard deviations.

suitable medium for the incubation of schistosomula obtained by transformation in supplemented HBSS. The parasites remained viable for 120 h with an average viability value of 2.5. On the other hand, all schistosomula had died by 72 hours in Basch medium and by 144 hours in DMEM (Figure 2).

Evaluation of resazurin as potential fluorimetric marker for the *S. haematobium* NTS *in vitro* assay

In an initial experiment the relationship between the fluorescence signal of the converted resazurin and schistosomula numbers (0–250 NTS tested) was studied. A linear relationship ($R^2 = 0.95$) between fluorescence development and NTS number was observed within a range of 25–200 NTS per well, as depicted in Figure 3. No increase in the fluorescence signal was observed using 250 schistosomula per well and above. Dead schistosomula and medium did not show any significant background signals.

In vitro S. haematobium NTS drug assay using microscopical readout compared with fluorimetric readout

As seen in Table 1, mefloquine showed the highest activity (IC_{50} value of 0.5 μM) followed by artesunate (1.4 μM), praziquantel (1.5 μM) and metrifonate (1.6 μM) against *S. haematobium* NTS using microscopical readout. For oxamniquine only low to moderate antischistosomal effects were observed against the schistosomular stage (IC_{50}: 26.5 μM). Severe effects on the morphology of *S. haematobium* NTS were observed 24, 48 and 72 hours post exposure with praziquantel, mefloquine and artesunate. Concentrations of 90 μg/ml praziquantel and mefloquine killed the parasite within 48 and 24 hours, respectively. Both drugs caused strong morphological changes such as severe deformation and increased granularity. Metrifonate killed all the parasites, after 24 hours drug exposure with 90 μg/ml and after 72 hours at a concentration of 30 μg/ml. At lower concentrations such as 1.1 μg/ml, 3.3 μg/ml and 10 μg/ml, motility was reduced until hardly any movement could be observed after 48 and 72 hours. Oxamniquine treatment caused only slight morphological changes such as rounding but resulted in strongly reduced motility.

In a next step, IC_{50} values were determined in this assay using resazurin as viability marker. Praziquantel revealed the highest activity with an IC_{50} below 3.5 μM ($P < 0.05$) (Table 1). Mefloquine (6.4 μM) and artesunate (7.4 μM) showed similar promising activities whereas oxamniquine (>322.2 μM) and metrifonate (348.3 μM) were characterized by a low activity in this assay (all $P < 0.05$).

The IC_{50} values obtained for praziquantel, mefloquine and artesunate against *S. haematobium* NTS presented within similar ranges when evaluated microscopically or fluorimetrically. Oxamniquine lacked an antischistosomal effect according to the fluorimetric readout ($IC_{50} > 322.2$ μM) while a moderate activity was observed when the effects of the drug were assessed microscopically (26.5 μM). To note, great differences were also observed between the readouts for metrifonate (348.3 μM versus 1.6 μM).

Comparison of IC_{50} values of *S. haematobium* and *S. mansoni* NTS

Using microscopy, very similar IC_{50} values of the 5 standard drugs were calculated for *S. haematobium* and *S. mansoni* NTS (Table 1).

Discussion

S. haematobium is a neglected parasite that still affects several million people per annum [1-4]. There is currently one drug available as the core treatment of schistosomiasis and it is worrying that low cure rates with praziquantel have already been reported in several endemic countries for *S. mansoni* [18]. To our knowledge,

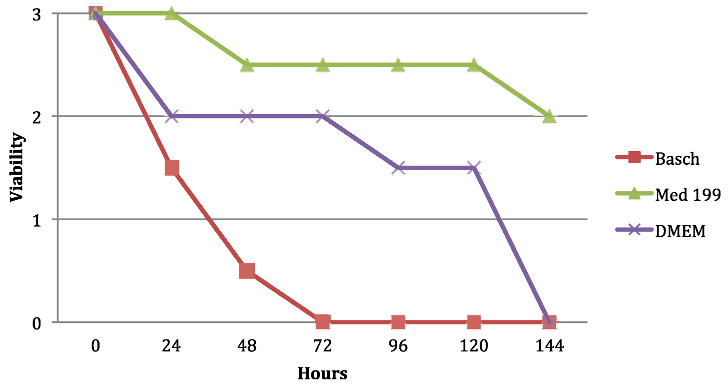

Figure 2 Survival time of *S. haematobium* NTS (150–200 NTS per well) in different culture media.

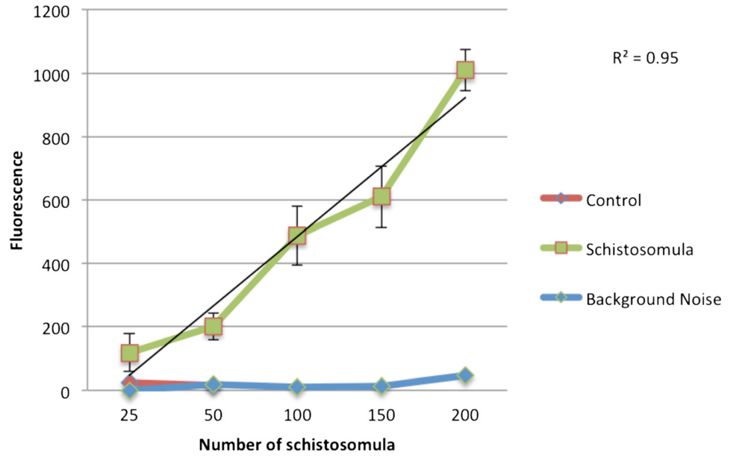

Figure 3 Relationship between fluorescence signal and number of schistosomula (25–250 schistosomula per well) in the resazurin based assay. Figure shows an average curve of four replicates, bars indicate standard deviations of the performed experiments. Fluorescence was measured after 24 hours of incubation. The fluorescence decreased with schistosomula concentrations above 200 per well. Linear regression is shown for data of 25-200 NTS.

we have for the first time established an *in vitro* drug sensitivity assay using *S. haematobium* NTS and additionally evaluated resazurin as a viability marker in this assay.

We first focused on the intermediate host snails and observed that the cercarial emergence rhythm of *B. glabrata* infected with *S. mansoni* as well as *B. truncatus* with *S. haematobium* showed one distinct peak for each species. It is important to state that these observations are based on long term laboratory isolates and the cercarial output of the imported *Bulinus* snails could also be influenced by the transportation between laboratories (~3 days of transport). However, the daily emergence pattern peak for *B. glabrata* occurred between 11 am and 1 pm, whereas for *B. truncatus* the peak occurred a little earlier between 10 am and 11.30 am. These results underline the fact that infected *B. glabrata* and *B. truncatus* snails show a distinct cercarial chronobiology [19,20]. It has been shown that *B. glabrata* shed approximately 2.7 times the number of cercariae per day (mean of 446 cercariae per snail) compared to *B. truncatus* (mean of 160 cercariae per snail), despite *B. truncatus* being exposed to a greater number of miracidia (10 per snail) than *B. glabrata* (8 per snail). We did not observe any influence of size or appearance of the snails such as calcareous deposit (calcification strips on the snail's shell) on the daily cercarial shedding in *B. truncatus* snails. Surprisingly, these findings contradict a previous report [21] that had shown that cercarial output is related to the size of snail, meaning larger snails (9–10 mm diameter) shed much more cercariae than smaller sized snails (5–6 mm).

Table 1 Comparison of the IC_{50} values of tested drugs for *S. haematobium* and *S. mansoni* schistosomula at the 72 hours time point evaluated microscopically and for *S. haematobium* NTS additionally fluorimetrically with resazurin

Technique	Drug	IC $_{50}$ values (μM) (mean ± SD)	
		S. haematobium	*S. mansoni*
Microscopy	Praziquantel	1.5 ± 1.1	0.7 ± 0.1
	Mefloquine	0.5 ± 0.2	0.7 ± 0.1
	Artesunate	1.4 ± 0.1	1.5 ± 0.6
	Metrifonate	1.6 ± 0.0	0.8 ± 0.1
	Oxamniquine	26.5 ± 3.4	11.8 ±0.0
Resazurin	Praziquantel	<3.5	ND
	Mefloquine	6.4	ND
	Artesunate	7.4	ND
	Metrifonate	348.3	ND
	Oxamniquine	>322.2	ND

IC_{50} values are calculated from three replicates at a concentration range of 1.1-90 μg/ml. ND: Not determined.

The mechanical transformation as described by Ramalho-Pinto *et al.* for *S. mansoni* was successfully tested on *S. haematobium* and yielded a transformation rate of 69% [11]. Overall both transformation methods, mechanical and chemical turned out to be very applicable on *S. haematobium* cercariae and were simple to perform. It is interesting to note that the chemical stimulation had a much lower transformation rate (30–41%) than the mechanical stimulation (59–87%). Salafsky *et al.* [22] reported that chemically transformed cercariae lost their osmoregulation ability at a much higher rate than during any other transformation method. Furthermore, Ramalho-Pinto *et al.* [11]

observed that tail-less cercariae have increased water sensitivity as compared to fully intact cercariae. These findings might explain the reason for the lower transformation rate in chemical stimulation.

We made the observation that increasing the time of vortexing resulted in decreased fitness of the parasites over time (data not shown). For this reason, we did not further increase the vortex time. We also did not use the syringe method, which might be worthwhile testing in future studies to achieve even higher transformation rates.

After successfully transforming cercariae, schistosomula had to be purified from the tails shed and fully intact cercariae. Unfortunately, the ice method described by Fred Lewis [14] turned out to be less successful with *S. haematobium* and thus two other purification methods were tested, namely the swirling method and Percoll® method, which were previously described in the literature [13,14]. Both of these methods successfully separated tails from the schistosomula. Percoll® turned out to be twice as effective as the swirling method. The silica colloid gradient was non-toxic and did not affect any assay procedures [13]. Schistosomula isolated by centrifugation on Percoll® did not show any loss in viability, as evaluated by morphological examination. However, it must be emphasized that using Percoll® for the purification is an expensive method compared to the swirling method. Nevertheless, taking all advantages of the gradient together, Percoll® was used for all further transformation and purification experiments performed.

Drug sensitivity screenings were performed on newly transformed *S. haematobium* schistosomula, derived from successful vortex transformations, first with the classical microscopical readout. Note that, the assessment of the parasite viability microscopically *in vitro* is based on two parameters: regular or lack of movement of larvae (motility) and morphological changes such as granularity and shape alterations [22]. However, microscopic assessment is subjective, slow, labor intensive and requires experience [23,24]. For this reason, it is crucial to find other evaluation and screening methods based not only on microscopic examination. In our study the widely used viability indicator resazurin, the active component of Alamar Blue®, was evaluated.

In our preliminary studies it could be shown that a linear correlation exists between the fluorescence signal and viable transformed schistosomula. Fluorescence values per well increased proportionally to the number of schistosomula up to the amount of 200 schistosomula. The use of more than 200 schistosomula resulted in decreased fluorescence values. This might be due to crowding effects in the well.

A correlation is observed between microscopic and fluorometric readout treating *S. haematobium* with artesunate, praziquantel and mefloquine. All three drugs showed clear dose response relationships (decrease in viability with increasing concentrations) in both the microscopic and fluorometric readout. However, the determined IC_{50} values in the resazurin based assay presented a slightly higher range compared to values calculated based on microscopy. This indicates that the sensitivity of resazurin as an indicator of metabolic activity, shows a higher sensitivity on the detection of viability than the motility observations via the microscope. Furthermore, it is important to mention that in particular for the microscopic results for several of the tested drugs, the lowest concentrations tested were in the range of the calculated IC_{50} values (e.g. mefloquine), and lower concentrations were not tested in the present work resulting in extrapolated IC_{50} values. On the other hand, important morphological criteria are taken into account within the microscopic approach, which might be missed using fluorimetric viability markers. Interestingly, the fluorometric readout regarding oxamniquine and metrifonate treatment showed that NTS were still alive and metabolically functioning but the observation of the treated parasites via the microscope showed strong changes on motility and slight morphological changes of the treated schistosomula. Summarizing, one can state that a fluorimetric drug assay with *S. haematobium* NTS based on resazurin is efficient in detecting severely damaged respectively dead schistosomula, as already described for *S. mansoni* NTS [10]. Unfortunately, the assay cannot detect drug effects on the motility of the worms or drugs with slight morphological damages as demonstrated for oxamniquine and metrifonate.

In our panel of known antischistosomal drugs, metrifonate and oxamniquine were included though both drugs have been replaced by praziquantel and are not used any longer [6]. Interestingly, we observed a good *in vitro* activity of metrifonate, a drug recommended for treating infections with *S. haematobium*, on *S. mansoni* NTS, which is in line with a previous study using adult worms [25]. Oxamniquine (used for the treatment of *S. mansoni* infections) showed moderate activity against *S. haematobium* and *S. mansoni* NTS, a finding, which is in contrast to previous results using *in vitro* cultures with adult worms [26]. *S. haematobium* adults were not affected by oxamniquine, while a moderate activity was observed against *S. mansoni* adult worms [15,26]. These varying drug sensitivities between the schistosomular and adult stage might possibly be explained with differences in drug activating enzymes, the main target of oxamniquine [26].

Conclusions

We have successfully developed an *in vitro* assay based on *S. haematobium* NTS. Using mechanical stress to transform cercariae into schistosomula instead of working with adult worms from mice and hamsters reduces the use of animals and thus stands in accordance with the 3Rs animal protection principles [9]. It should be highlighted that although schistosomula have great advantages when used for viability assays, it is crucial not to neglect adult schistosomes, as previously mentioned, differing susceptibilities on different parasite stages are not uncommon. Nonetheless schistosomula serve as an important starting point for the assessment of new drug candidates since it allows carrying out medium to high-throughput screenings.

The resazurin-based assay has proven to be effective, simple and possibly useful for large screening of drugs against *S. haematobium* NTS, though compounds acting solely on the motility or morphology of the worms (metrifonate, oxamniquine) might be missed.

However, it must be mentioned that working with *S. haematobium* schistosomula respectively, *B. truncatus* snails was more time consuming than working with *S. mansoni/ B. glabrata*. In addition to that, drug sensitivity screening for both species showed very similar results. Nevertheless, *S. haematobium* must not be neglected despite its more laborious work. *S. haematobium* infections still occur in most parts of Africa and are a major problem in developing countries [27]. For this reason, it is crucial to continue working with both species, possibly with *S. mansoni* first followed by testing of active compounds against *S. haematobium*.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

MM, KI and JK designed the studies. MM carried out the experiments and wrote the first draft of the manuscript. KI and KJ revised the manuscript. All authors read and approved the final version of the manuscript.

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Chapter 3

Preclinical studies with antimalarials

3.1 Antischistosomal activities of mefloquine-related arylmethanols

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Antischistosomal Activities of Mefloquine-Related Arylmethanols

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Interesting antischistosomal properties have been documented for the antimalarial mefloquine, a 4-quinolinemethanol. We evaluated the antischistosomal activities of nine mefloquine-related compounds belonging to the 4-pyridinemethanols, 9-phenanthrenmethanols, and 4-quinolinemethanols. Eight compounds revealed high activities against *Schistosoma mansoni in vitro*, with two drugs (the 4-quinolinemethanols WR7573 and WR7930) characterized by significantly lower half-maximal inhibitory concentrations (IC_{50}s) (2.7 and 3.5 μM, respectively) compared to mefloquine (11.4 μM). Mefloquine and WR7930 showed significantly decreased IC_{50}s when incubated in the presence of hemoglobin. High worm burden reductions (WBR) were obtained with enpiroline (WBR, 82.7%; dosage, 200 mg/kg of body weight) and its *threo* isomers (+)-*threo* (WBR, 100%) and (−)-*threo* (WBR, 89%) and with WR7930 (WBR, 87%; dosage, 100 mg/kg) against adult *S. mansoni* in mice. Furthermore, excellent *in vitro* and *in vivo* antischistosomal activity was observed for two WR7930-related structures (WR29252 and WR7524). In addition, mefloquine (WBR, 81%), enpiroline (WBR, 77%), and WR7930 (WBR, 100%) showed high activities against *S. haematobium* harbored in mice following single oral doses of 200 mg/kg. These results provide a deeper insight into the structural features of the arylmethanols that rule antischistosomal activity. Further studies should be launched with enpiroline and WR7930.

Human schistosomiasis is a neglected tropical disease whose burden is mainly concentrated in sub-Saharan Africa and affects approximately 207 million people (30). The three main schistosome species parasitizing humans are *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum*. Today, treatment with praziquantel is the core component of schistosomiasis control programs (32, 34, 35). There is no question that heavy reliance on a single drug bears a risk of drug resistance development. Indeed, in different regions of endemicity, lower cure rates have already been observed (9). An additional disadvantage of praziquantel is its stage-dependent susceptibility, showing only poor efficacy against immature schistosome stages (23). Therefore, drug discovery in the field of schistosomiasis remains an important task. Antischistosomal properties of the antimalarial drug mefloquine were first mentioned in 2008, when it was shown that a dosage of 150 mg/kg of body weight significantly reduced the egg burden in *S. mansoni*-infected mice (33). Further investigations revealed that mefloquine possesses good *in vivo* efficacy, with a single oral dosage of 200 mg/kg resulting in a total worm burden reduction of 72.3% in *S. mansoni*-infected mice. Another interesting characteristic of mefloquine is its efficacy against the juvenile immature stage (13). Finally, a randomized clinical trial that investigated the effect of mefloquine and mefloquine-artesunate in *S. haematobium*-infected patients was recently performed. It was shown that a combination therapy of mefloquine-artesunate resulted in moderate cure and high egg reduction rates (15).

Based on these promising findings, we were motivated to test mefloquine-related compounds belonging to the three major groups of arylmethanols well described in antimalarial research, namely, 4-quinolinemethanols, 9-phenanthrenmethanols, and 4-pyridinemethanols (4), in order to elucidate their potential as antischistosomal lead candidates. In addition, a study on mefloquine-related compounds might provide us with a deeper understanding of structural features needed for antischistosomal activity of arylmethanols. The selected nine arylmethanols were first tested against *S. mansoni* schistosomula and adults *in vitro*. Promising compounds were followed up *in vivo*. Candidates characterized by high *in vivo* activity against *S. mansoni* were tested against *S. haematobium*. In addition, promising candidates were incubated in the presence of hemoglobin, hemin, or red blood cells to compare drug activities in relation to the postulated mechanistic heme dependency of arylmethanols (6). Finally, isothermal microcalorimetry (IMC) was used to investigate the antischistosomal properties of lead candidates in greater detail and to compare their levels of activity with mefloquine.

MATERIALS AND METHODS

Drugs and media. The following 11 arylmethanols were kindly provided by the Walter Reed Army Institute of Research (WRAIR): compounds 1 (WR171669; halofantrine), 2 (WR178460; *n*-desbutylhalofantrine), 3 [WR190420; 1-(1,3-dichloro-6-trifluoromethyl-9-phenanthryl)-2-(2-piperidyl)ethanol], 4 [WR148946; 1-(2,6-bis(4-(trifluoromethyl)phenyl)pyridine-4-yl)-2-(dibutylamino)ethanol], 5 [WR151312; 1-(2,6-bis(4-(trifluoromethyl)phenyl)pyri-dine-4-yl)-2-(butylamino)ethanol], 6 [WR154904; (2,6-bis(4-(trifluoromethyl)phenyl)pyridine-4-yl)(piperidin-2-yl)methanol], 7 (WR180409; enpiroline), 8 {WR7573; (2-(4-chlorophenyl)benzo[*h*]quinolin-4-yl)(piperidin-1-yl)methanol}, 9 [WR7930; (6,8-dichloro-2-phenylquinolin-4-yl)(piperidin-1-yl)methanol], 12 [WR29252; 6,8-dichloro-2-(4′-chlorophenyl)-alpha-(di-n-butylaminomethyl)-4-quinoline methanol], and 13 [WR7524; alpha-butylaminomethyl-2-(4′-chlorophenyl)-6,8-dichloro-4-quinoline methanol]. Additionally, we received the two *threo* enantiomers of enpiroline (compounds 10 [WR247733] and 11 [WR247734]) from the WRAIR. Mefloquine was kindly provided by Mepha AG (Aesch, Switzerland). The chemical structures of the arylmethanols investigated are shown in Fig. 1 to 3. For the *in vitro* studies, all drugs were dissolved in stock solutions of

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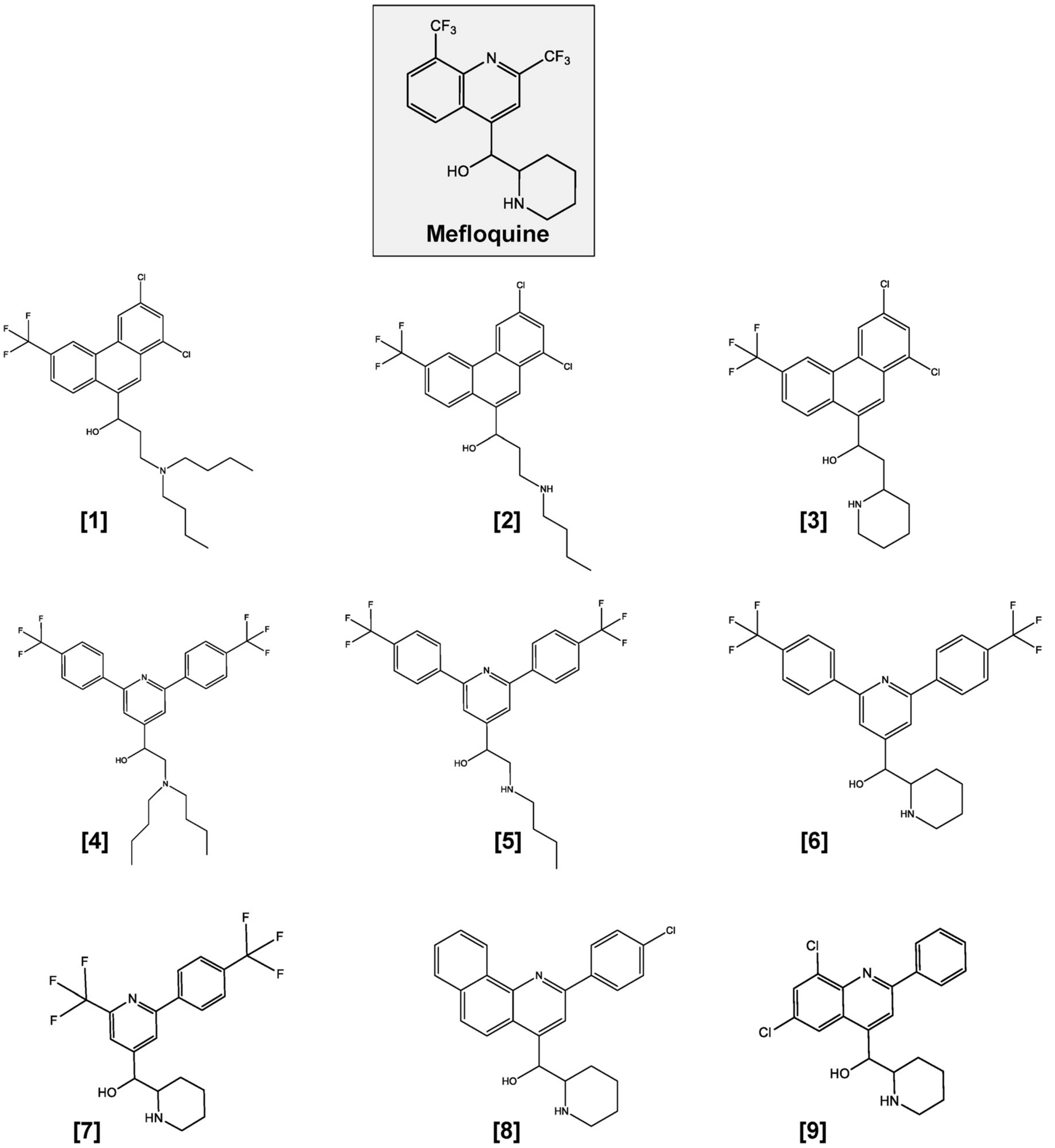


FIG 1 Chemical structures of the arylmethanols investigated: halofantrine (compound 1); *n*-desbutylhalofantrine (compound 2); WR190420 (compound 3); WR148946 (compound 4); WR151312 (compound 5); WR154904 (compound 6); enpiroline/WR180409 (compound 7); WR7573 (compound 8); WR7930 (compound 9).

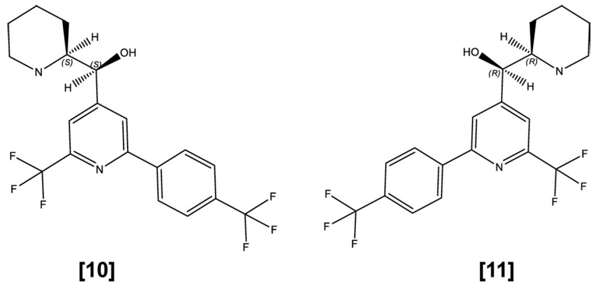
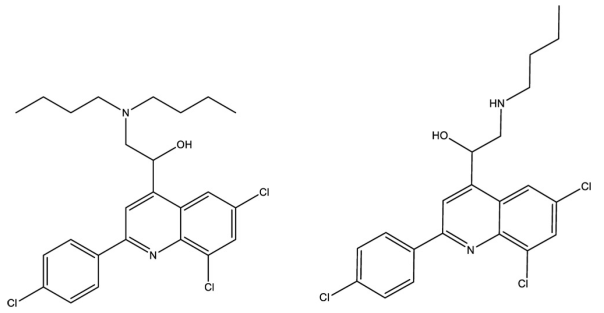


FIG 2 Chemical structures of the enantiomers of enpiroline: (−)-*threo* enpiroline (compound 10); (+)-*threo* enpiroline (compound 11).

dimethyl-sulfoxide (DMSO) (Sigma-Aldrich Chemie GmbH) (10 mg/ml). For the *in vivo* studies, drugs were freshly prepared as water-based suspensions in 7% (vol/vol) Tween 80 and 3% (vol/vol) ethanol before *per os* administration to animals.

The hemin solution (1.5 mM) was prepared as follows: 50 mg of hemin-chloride (Fluka Analytical, Netherlands) was dissolved in 10 ml of 0.1 M NaOH and 39.5 ml of phosphate-buffered saline (PBS), and 0.5 ml of 1 M HCl was added to adjust the pH to ~7.4. The hemoglobin solution (0.23 mM) was prepared using 750 mg of hemoglobin from bovine blood (Sigma-Aldrich) dissolved in identical amounts of NaOH, PBS, and HCl as described above. Supplemented RPMI media were achieved by adding 8% of freshly prepared hemin solution (final concentration of 120 μM),



[12] [13]

FIG 3 Chemical structures of the WR7930-related compounds WR29252 (compound 12) and WR7524 (compound 13).

10% of prepared hemoglobin solution (final concentration of 23 μM), or 2% red blood cells (RBC) from concentrate (group A; Rh positive).

Animals and parasites. All animal studies were conducted at the Swiss Tropical and Public Health Institute (Basel, Switzerland) following Swiss national and cantonal regulations on animal welfare (permission no. 2070). Three-week-old (weight, ca. 14 g) female NMRI mice were purchased from Charles River (Sulzfeld, Germany) or Harlan Laboratories (Blackthorn, United Kingdom). Three-week-old Syrian golden hamsters were purchased from Charles River. All animals were allowed to adapt for 1 week under controlled conditions (temperature, ca. 22°C; humidity, ca. 50%; 12-h light and 12-h dark cycle; free access to rodent diet and water) before initiation of experiments. Mice were infected subcutaneously with 80 to 100 *S. mansoni* (Liberian strain) cercariae. Cercariae were harvested from infected intermediate *Biomphalaria glabrata* host snails by exposure to light for 3 h, following the standard procedures of our laboratory. The *S. haematobium* (Egyptian strain) infection was performed using *Bulinus truncatus truncatus* snails (kindly provided by the Biomedical Research Institute, Rockville, MD). Mice and hamsters were subcutaneously infected with 300 to 350 and with 120 *S. haematobium* cercariae, respectively.

***In vitro* studies with *S. mansoni*. (i) Preparation of NTS.** Cercariae of *S. mansoni* were mechanically transformed into newly transformed schistosomula (NTS) (12, 18). The concentration of the obtained schistosomulum suspension was adjusted to 100 NTS per 50 μl with Medium 199 (Invitrogen, Carlsbad, CA) supplemented with 5% heat-inactivated fetal calf serum (iFCS), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA). NTS suspensions were incubated at 37°C in an atmosphere of 5% CO_2 in ambient air for a minimum of 12 to 24 h until usage, ensuring completed conversion from cercariae to NTS (8).

(ii) Preparation of adult schistosomes. At 7 to 8 weeks postinfection with *S. mansoni*, NMRI mice were sacrificed by administration of CO_2 and dissected, and adult worms were harvested from hepatic portal veins and mesenteric veins (36). Schistosomes were placed in RPMI 1640 culture medium supplemented with 5% heat-inactivated fetal calf serum (iFCS), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA) at 37°C in an atmosphere of 5% CO_2 until use. Likewise, adult flukes were collected from *S. haematobium*-infected NMRI mice; however, they were harvested 12 to 14 weeks postinfection.

(iii) Drug sensitivity assays with NTS. Drugs were tested at a concentration range of 0.37 to 90 μg/ml (0.37, 1.1, 3.3, 10, 30, and 90 μg/ml). For that purpose, flat-bottom 96-well plates (BD Falcon) were prepared with Medium 199 supplemented with iFCS and antibiotics. Appropriate drug dilutions followed by the NTS suspension, containing 100 NTS per 50 μl, were added to each well to achieve a total volume of 250 μl per well. The highest DMSO concentration used, diluted in Medium 199, served as a control. Plates were incubated at 37°C in an atmosphere of 5% CO_2. Based on microscope readouts (Carl Zeiss, Germany; magnification, ×80), NTS were evaluated with regard to death, changes in motility, viability, and morphological alterations at three different time points (24, 48, and 72 h post-drug exposure). Drug effects were evaluated using a viability scale, as described previously (12, 18). Briefly, parasite fitness, morphology, and motility were classified with scores ranging from 4 (hyperactivity, increased motility) and 3 (normal activity, no morphological changes) to 0 (all worms dead). Each concentration was tested in duplicate, and experiments were repeated at least three times. Motility scale values obtained at the 72-h time point were used to determine half-maximal inhibitory concentrations (IC_{50}s) of the individual drugs using CompuSyn software (version 3.0.1 [2007]; ComboSyn, Inc.).

(iv) Drug sensitivity assay with adult schistosomes. For the *in vitro* screening of adult flukes, RPMI 1640 medium, supplemented with iFCS and antibiotics, was placed into flat-bottom 24-well plates (BD Falcon). Dilution series were performed with DMSO stock solutions (concentration, 10 mg/ml) to obtain final concentrations of each test drug of 1, 3.3, 10, 30, and 90 μg/ml in a final volume of 1.4 ml per well. Three worms of both sexes were placed into each well. Experiments investigating the influence of iron-containing products were carried out with both schistosoma worms, which were incubated in supplemented RPMI media for 7 days without addition of RBC or any other additional iron source before usage, and freshly dissected worms. Wells with the highest concentration of DMSO in medium served as controls. Phenotypes were monitored after 24, 48, and 72 h using the motility scale described by Ramirez et al. (25) and an inverse microscope (Carl Zeiss, Germany; magnification, ×80). Each experiment was performed at least three times. IC_{50}s were calculated with CompuSyn software as described above for NTS.

In vivo studies with *S. mansoni* and *S. haematobium*. Groups of 3 to 5 infected NMRI mice or 4 Syrian golden hamsters characterized by a patent schistosome infection (49 days for *S. mansoni* and 90 days for *S. haematobium*) were treated orally with the test drug using single doses (100 or 200 mg/kg of body weight). Seven or eight ($n = 12$ for *S. haematobium* infection) untreated mice or four hamsters served as controls. At 14 days posttreatment, animals were sacrificed by the CO_2 method and dissected. Worms were sexed and counted (36). Worm burdens of treated mice were compared to those of control animals and reductions of worm burden calculated.

IMC drug assay with adult *S. mansoni*. Two lead candidates (enpiroline and WR7930), characterized by high WBR, were further characterized and compared to mefloquine using an isothermal microcalorimeter as described by Manneck et al. (17). Briefly, schistosome heat production and motility data (derived from noise amplitudes) were measured using a 48-channel isothermal microcalorimeter (model TAM 48; TA Instruments, New Castle, DE) over a time period of 120 h. Samples were prepared in glass ampoules with 2,900 μl of medium (supplemented RPMI 1640) containing 3 or 4 adult worms. Prewarmed (37°C) ampoules were placed in channels, and equilibration was performed for 5 h until a stable signal was observed. Drug suspensions (at a concentration of 30, 300, or 900 μg/ml) in supplemented medium (volume of 100 μl) were injected, using 1-ml syringes (BD Plastipak; Becton, Dickinson S.A., Madrid, Spain) to reach final concentrations (1, 10, and 30 μg/ml per ampoule). Ampoules with dead worms served as negative controls, and ampoules with worms treated with the highest concentration of DMSO served as positive controls. The heat flow was recorded as 1 data point per min over at least 120 h. Experiments using each concentration were repeated at least three times.

Statistics. Parasite motility of treated and untreated NTS and adult flukes were calculated as means (± standard deviation) using Microsoft Excel software. IC_{50}s were determined using the CompuSyn software. To compare the IC_{50}s of lead candidates determined in various media, the Kruskal-Wallis test was performed (results were considered significant at $P \leq 0.05\%$) (StatsDirect statistical software, version 2.7.2; StatsDirect Ltd., United Kingdom). For *in vivo* studies, the Kruskal-Wallis test was also utilized, to compare the medians of the responses of the treatment

TABLE 1 *In vitro* IC$_{50}$s of selected arylmethanols for NTS and adult *S. mansoni* 72 h posttreatment[a]

		IC$_{50}$ (SD) [μM]	
Methanol group	Compound	NTS	Adult worms
4-Quinoline	Mefloquine	6.1 (1.3)	11.4 (6.4)
9-Phenanthrene	1 (WR171669)	1.9 (0.4)	7.2 (1.6)
	2 (WR178460)	0.2 (0.2)	7.1 (6.4)
	3 (WR190420)	3.0 (2.6)	5.4 (1.7)
4-Pyridine	4 (WR148946)	12.1 (8.2)	>52.2
	5 (WR151312)	2.7 (3.1)	4.0 (3.4)
	6 (WR154904)	0.2 (0)	5.3 (6.2)
	7 (WR180409)	0.9 (0.2)	6.1 (4.7)
4-Quinoline	8 (WR7573)	1.4 (1.3)	2.7 (0.2)
	9 (WR7930)	0.2 (0.3)	3.5 (1.6)

[a] Experiments were carried out 3 times in duplicate for NTS ($n = 6$) and 3 times for adult schistosomes ($n = 3$) for each tested drug. SD, standard deviation.

and control groups. A difference in median values was considered to be statistically significant at a level of 5%. Noise amplitudes and heat flows observed in calorimetric *in vitro* assays with adult *S. mansoni* were analyzed using R software and Microsoft Excel. As described by Manneck et al. (16), noise amplitude values follow an exponential decay. Endpoints of worm motility were determined by the intersection of the sample amplitude curve with the background signal noise of dead worms. The statistical significance of the means of heat flows (calculated with Microsoft Excel) was assessed using the parametric paired *t* test at a 5% level of significance (StatsDirect statistical software, version 2.7.2; StatsDirect Ltd., United Kingdom).

RESULTS

***In vitro* activities of selected arylmethanols against NTS and adult *S. mansoni*.** IC$_{50}$s of nine compounds tested against NTS and adult *S. mansoni* worms are summarized in Table 1. With exception of one drug (compound 4 [WR148946]; IC$_{50}$ of 12.1 μM), all compounds tested showed higher activities (IC$_{50}$s of 0.2 to 3.70 μM) than mefloquine (IC$_{50}$ of 6.1 μM) against NTS. Compounds 1, 2, and 6 to 9 showed significantly higher activity than mefloquine. The highest activity against NTS was observed with compounds 2, 6, and 9 (IC$_{50}$s of 0.2 μM). A similar finding was obtained for adult *S. mansoni*. Eight compounds revealed higher activities against the adult stage of the parasite than mefloquine (IC$_{50}$ of 11.4 μM). Two compounds (compounds 8 and 9) revealed significantly lower IC$_{50}$s (2.7 and 3.5 μM) than mefloquine. Compounds showing increased activity compared to mefloquine against adult *S. mansoni* were followed up *in vivo*.

***In vivo* activity of arylmethanols against adult *S. mansoni*.** *In vivo* activities of eight arylmethanols are summarized in Table 2. Previous studies have reported worm burden reductions of 45.8% and 64.0% when a single oral dose of mefloquine at 100 mg/kg of body weight was administered to *S. mansoni*-infected mice (13, 14). At 100 mg/kg, compound 6 completely lacked activity. A low total worm burden reduction of 18.9% was observed for compound 5. Moderate total worm burden reductions of 37.1%, 40.5%, and 52.0% were achieved with compounds 3, 8, and 2, respectively. Significant total worm burden reductions of 58.5% ($P = 0.02$) and 60.4% ($P = 0.01$) (and female worm burden reductions of 58.6% and 68.6%) were documented for halofantrine and enpiroline, respectively. The highest activity (total and female worm burden reductions of 87.0% and 91.9%, respectively [$P = 0.01$]) was documented for compound 9 (WR7930).

Halofantrine was not considered further, since, in a previous study, an increased dose (400 mg/kg) achieved only a moderate total worm burden reduction of 51.7% (13). Enpiroline, an antimalarial drug candidate that had already been tested in phase 2 trials (7), and its enantiomers (compounds 10 and 11) were selected for further studies along with WR7930 and two closely related molecules (compounds 12 and 13).

***In vitro* activity of WR7930, enpiroline, and mefloquine against adult *S. mansoni* determined using IMC.** The effects of the lead candidates enpiroline, WR7930, and mefloquine on the heat flow and motility of adult *S. mansoni* determined using three

TABLE 2 Effect of selected arylmethanols on worm burden in mice harboring adult *S. mansoni* (patent infection, 49 days), stratified by sex and worm distribution[a]

				Mean no. of worms (SD)							
Compound (100 mg/kg)	No. of mice investigated	No. of mice that died	No. of mice cured	Liver	Mesenteric veins	Total	Females	TWR (%)	*P* value	FWBR (%)	*P* value
Control	8			0.6 (1.0)	13.0 (3.0)	13.9 (2.5)	6.4 (2.5)				
5	4	0	0	0.8 (0.4)	10.5 (5.2)	11.3 (5.1)	4.8 (2.2)	18.9	0.3	25.5	0.35
6	3	0	0	0 (0)	21.3 (12.5)	21.3 (12.5)	10.3 (6.1)	0	0.92	0	0.84
7	6	2	0	0.5 (0.9)	5.0 (3.5)	5.5 (3.2)	2.0 (1.9)	60.4	0.01	68.6	0.01
8	4	0	0	1.0 (1.0)	7.3 (4.5)	8.3 (4.5)	4.0 (2.9)	40.5	0.03	37.3	0.2
Control	7			0.3 (0.8)	24.9 (7.6)	25.0 (8.1)	12.3 (3.9)				
2	4	1	0	0.5 (0.5)	11.5 (3.9)	12.0 (6.0)	5.5 (2.5)	52.0	0.11	55.4	0.09
9	4	0	0	2.3 (0.8)	1.0 (1.7)	2.3 (0.8)	1.0 (0.7)	87.0	0.01	91.9	0.01
Control	6			1.3 (1.0)	30.0 (3.9)	31.3 (3.7)	14.5 (3.5)				
1	3	0	0	1.0 (2.3)	11.7 (6.2)	13.0 (5.8)	5.0 (1.7)	58.5	0.02	58.6	0.02
Control	9			2.0 (3.5)	27.0 (27.7)	29.0 (28.5)	17.8 (24.1)				
3	4	0	0	0.8 (1.5)	16.0 (11.5)	18.3 (10.9)	7.8 (4.5)	37.1	0.76	67.9	0.76
MQ (data from reference 13)								45.8		56.3	
MQ (data from reference 14)								64.0		77.6	

[a] SD, standard deviation; TWR, total worm burden reduction; FWBR, female worm burden reduction; MQ, mefloquine.

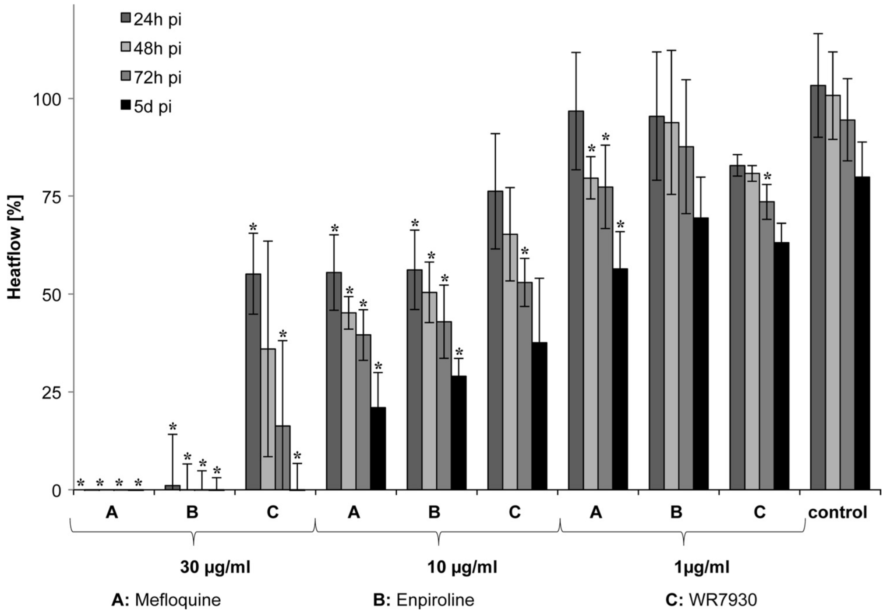


FIG 4 Heat flow of schistosomes recorded over 5 days (d) posttreatment with (A) mefloquine, (B) enpiroline, or (C) WR7930 at concentrations of 30, 10, and 1 μg/ml. Bars indicate standard deviations. Experiments were conducted in quadruplicate with 4 worms each. Asterisks (*) indicate heat flow values which are significantly ($P \leq 0.05$) lower than control values obtained with untreated worms. pi, postinjection.

different concentrations (1, 10, and 30 μg/ml) were compared using IMC over a period of 5 days.

Enpiroline and mefloquine showed similar effects on the heat flow (Fig. 4) and motility of adult *S. mansoni*, which is consistent with the microscopically obtained results. Concentrations of 30 and 10 μg/ml of each drug resulted in immediate losses of motility and a significant decrease of heat flow. The lowest tested concentration (1 μg/ml) of both drugs had only a minor effect on the motility (all worms were active for >5 days). At this concentration, however, the heat flow was significantly decreased compared to that seen with the control at 48 h post-mefloquine treatment whereas enpiroline administration did not result in a significant decrease in heat flow. Heat flow and motility were less affected by WR7930. A significant heat flow reduction at 5 days posttreatment with WR7930 was observed only with the highest concentration (30 μg/ml). Motility loss was documented 19.3 h posttreatment at the highest concentration (30 μg/ml).

***In vitro* and *in vivo* activity of enpiroline and its enantiomers against *S. mansoni*.** The two enantiomers of enpiroline (structures shown in Fig. 2) showed similar antischistosomal activities *in vitro* as determined using a microscopic readout (Table 3). The ratio of the microscopically evaluated IC_{50} of the (−) isomer (9.5 μM) to the IC_{50} of the (+) isomer (9.0 μM) against adult *S. mansoni* is 1.06. Slightly higher activities against NTS (0.9 μM) and adult *S. mansoni* worms (6.1 μM) were observed with enpiroline.

IMC revealed that, at a concentration of 20 μM, the (−)-*threo* isomer achieved a loss of movement of schistosomes 27 h posttreatment compared to 46.5 h for the (+)-*threo* enantiomer. On the other hand, at a concentration of 20 μM, enpiroline inhibited the motility of adult *S. mansoni* immediately after exposure.

The *in vivo* efficacies obtained with enpiroline and its *threo* enantiomers at 200 mg/kg are summarized in Table 4. No significant differences in activity between the two enantiomers and enpiroline could be observed. *In vivo* treatment of mice with the (+)-*threo* enantiomer resulted in complete elimination of worms. However, 4 out of 5 mice died following treatment with this drug. Highly significant total and female worm burden reductions were observed following treatment of *S. mansoni*-infected mice with the racemate enpiroline (82.7% and 83.8%, respectively) and the (−) isomer (89.0% and 91.9%, respectively) at 200 mg/kg. Based on these findings, the *in vitro* cytotoxicity was assessed using two different cell lines (HepG2 and L6 rat skeleton cells). The lead compounds (WR7930, enpiroline, and both enantiomers) showed patterns of cytotoxicity similar to that seen with the parent drug mefloquine. Hepatic HepG2-cells were strongly affected after treatment with the highest concentration (30 μg/ml), whereas the other tested concentrations (3 and 0.3 μg/ml) did not reduce the cell viability. After a 72-h treatment of L6 rat skeleton cells, IC_{50}s ranging from 9.7 to 16.5 μM were observed. Detailed *in vitro* cytotoxicity data are summarized in Tables S1 and S2 in the supplemental material.

TABLE 3 *In vitro* activity of enpiroline (*threo* racemate) and its (−)-*threo*/(+)-*threo* enantiomers against NTS and adult *S. mansoni* 72 h posttreatment as assessed microscopically[a]

	IC_{50} (SD) [μM]	
Compound	NTS	Adult worms
Enpiroline	0.9 (0.2)	6.1 (4.7)
(−)-*threo*	3.6 (4.7)	9.5 (6.5)
(+)-*threo*	2.8 (1.7)	9.0 (5.9)

[a] Experiments were carried out 3 times in duplicate for NTS ($n = 6$) and 3 times for adult schistosomes ($n = 3$) for each tested drug. SD, standard deviation.

TABLE 4 Effect of single oral doses of enpiroline and its (+)-*threo* and (−)-*threo* enantiomers at 200 mg/kg on worm burden in mice harboring adult *S. mansoni* (patent infection, 49 days), stratified by sex and worm distribution[a]

				Mean no. of worms (SD)							
Compound	No. of mice investigated	No. of mice that died	No. of mice cured	Liver	Mesenteric veins	Total	Females	TWR (%)	*P* value	FWBR (%)	*P* value
Control	7			0.3 (0.8)	24.9 (7.6)	25.0 (8.1)	12.3 (3.9)				
(−)-*threo*	4	0	0	1.3 (0.4)	1.3 (0.9)	2.8 (1.1)	1.0 (0.7)	89.0	0.01	91.9	0.01
(+)-*threo*	5	4	1	0	0	0	0	100.0		100.0	
Enpiroline	4	1	1	0.7 (0.9)	3.7 (1.9)	4.3 (4.2)	2.0 (2.2)	82.7	0.02	83.8	0.02
MQ (data from reference 13)								72.3		93.0	

[a] SD, standard deviation; TWR, total worm burden reduction; FWBR, female worm burden reduction; MQ, mefloquine.

***In vitro* and *in vivo* activities of WR7930-related compounds.** Since WR7930 showed very good *in vivo* antischistosomal activity at an oral dosage of 100 mg/kg in mice, two related structures featuring desbutyl and dibutyl aminostructures (Fig. 3) were investigated *in vitro* and *in vivo*. As shown in Table 5, desbutyl compound 13 showed lower *in vitro* activity against NTS (IC_{50}, 4.3 μM) and similar activity against adult schistosomes (IC_{50}, 3.9 μM) compared to WR7930 and higher activity than mefloquine on both stages. On the other hand, compound 12, a dibutyl analogue, showed lower activity than WR7930 against NTS (IC_{50}, 5.7 μM) and even less activity than mefloquine against adult *S. mansoni* (IC_{50}, 27.9 μM) (Table 5). Both compounds showed high antischistosomal *in vivo* activity, achieving total worm burden reductions of 75.0% (compound 12) and 81.9% (compound 13).

Influence of hemin, hemoglobin, or red blood cells on drug activity against adult schistosomes. As shown in Fig. 5 IC_{50}s of enpiroline did not differ significantly following supplementation of RPMI medium with hemin, hemoglobin, or RBC (RPMI, 6.1 μM; hemin, 8.0 μM; hemoglobin, 5.0 μM; RBC, 7.5 μM) ($P > 0.05$). Mefloquine revealed similar activities in RPMI medium, RPMI medium plus hemin, and RPMI medium plus RBC (11.4, 9.7, and 9.9 μM, respectively) ($P > 0.05$). A significantly 57-fold-lower IC_{50} (0.2 μM) was determined for mefloquine in the presence of hemoglobin compared to standard culture medium conditions ($P < 0.03$). Similarly, the highest antischistosomal activity of WR7930 was calculated in the presence of hemoglobin (0.2 μM). An 18-fold-higher IC_{50} (3.5 μM) was observed when adult schistosomes were exposed to WR7930 in RPMI medium followed by incubation in the presence of RBC (IC_{50}, 6.0 μM) and hemin (IC_{50}, 11.3 μM) ($P < 0.05$). All female worms died 4 h post-WR7930 exposure at the highest concentration (30 μg/ml), whereas males often stayed alive until 6 to 24 h postexposure. This effect could be observed in all investigated media.

No differences in results were observed in all experiments using starved or freshly dissected *S. mansoni*.

***In vivo* activity of enpiroline, WR7930, and mefloquine against *S. haematobium*.** Enpiroline, mefloquine, and WR7930 were studied in *S. haematobium*-infected mice and hamsters. *In vivo* findings are summarized in Table 6.

Mefloquine showed good activity against *S. haematobium in vivo*. Worm burden reductions of 61.9% and 93.7% were observed at dosages of 100 and 200 mg/kg, respectively, in hamsters. Similarly, a worm burden reduction of 81.3% was observed in *S. haematobium*-infected mice treated with a single dose at 200 mg/kg. Enpiroline showed comparable activity, with a worm burden reduction of 76.6% at a dose of 200 mg/kg in *S. haematobium*-infected mice. Complete elimination of worms was achieved by treating *S. haematobium*-infected mice with WR7930 at 200 mg/kg.

DISCUSSION

Mefloquine possesses a compelling antischistosomal prototype and might therefore serve as a starting point to identify one or more related lead compounds with high antischistosomal efficacy.

Nine compounds, representatives of 3 chemical classes, the 4-quinolinemethanols, 9-phenanthrenmethanols, and 4-pyridinemethanols, served as initial sources in our small structure-activity-relationship study. To our knowledge, compounds belonging to the 4-pyridinemethanols had not been tested against schistosomes to date. We selected four representatives of this group, including enpiroline, since the drug is characterized by good pharmacokinetic features and has already undergone clini-

TABLE 5 *In vitro* activity of WR7930-related compounds against NTS and adult *S. mansoni* and its *in vivo* effect on *S. mansoni* in infected NMRI mice treated with an oral dosage of 100 mg/kg[a]

	In vitro IC_{50} (SD) [μM]		*In vivo*								
					Mean no. of worms (SD)						
Treatment group	NTS	Adult worms	No. of mice investigated	No. of mice cured	Liver	Mesenteric veins	Total	TWR (%)	*P* value	FWBR (%)	*P* value
Control			9		2.0 (3.5)	27.0 (27.7)	29.0 (28.5)				
Compound 12	5.7 (3.3)	27.9 (6.8)	4	0	1.5 (1.0)	5.8 (2.6)	7.3 (2.8)	75.0	0.31	83.1	0.18
Compound 13	4.3 (2.2)	3.9 (0.7)	4	0	2.8 (2.8)	3.3 (2.5)	5.3 (1.5)	81.9	0.12	93.0	0.02

[a] *In vitro* experiments were carried out 3 times in duplicate for NTS ($n = 6$) and 3 times for adult schistosomes ($n = 3$) for each tested drug. SD, standard deviation; TWR, total worm burden reduction; FWBR, female worm burden reduction.

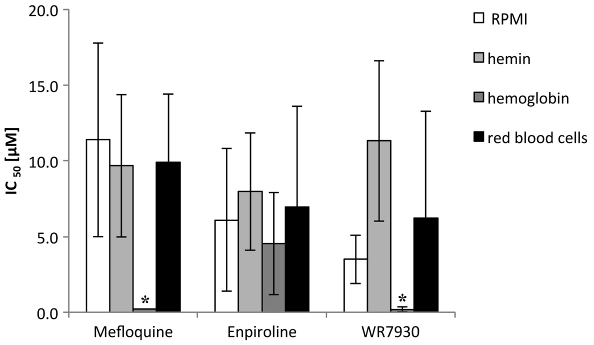


FIG 5 IC_{50}s calculated following exposure of adult *S. mansoni* worms to mefloquine, enpiroline, or WR7930 using different culture media (RPMI medium or RPMI medium supplemented with 120 μM hemin, 23 μM hemoglobin, or 2% RBC). Error bars indicate standard deviations of experiments ($n = 5$). Asterisks (*) indicate IC_{50}s which are significantly ($P \leq 0.05$) lower than the control values determined using RPMI medium.

cal testing against malaria in humans (3, 7). Three compounds from the 9-phenanthrenmethanols, halofantrine, *n*-desbutylhalofantrine (the main human metabolite of halofantrine), and a derivative characterized by a piperidyl (compound 3) similar to mefloquine, were chosen. Finally, two 4-quinolinemethanol analogs of WR7930 complemented our study.

Our findings confirm the potential of arylmethanols as lead structure candidates. Eleven compounds revealed high activities *in vitro*. In addition, 5 of 12 selected compounds showed *in vivo* activity in *S. mansoni*-infected NMRI mice that, relative to the activity of mefloquine, was comparable [enpiroline and its (−)-*threo* enantiomer] or even increased (WR7930 and its related structures WR29252 and WR7524). Activity was observed among all chemical subclasses, and no final conclusion on the structural needs for an ideal antischistosomal arylmethanol can be drawn.

The similarity of the susceptibilities seen with arylmethanols tested against *Plasmodium* and *Schistosoma* species is worth highlighting. When the tested 4-pyridinemethanols (compounds 4 to 7) were ranked in the order of their activities, a pattern was observed in our study that was similar to that observed in studies carried out in *Plasmodium falciparum*-infected owl monkeys (28). In more detail, enpiroline was found to be the most active representative followed by compound 5 showing moderate activity and compound 6 showing only minimal activity against *P. falciparum*. A similar trend was also observed with activity against *S. mansoni*. In addition, in our study, halofantrine and its *n*-desbutyl derivative showed IC_{50}s in similar ranges, a result that is in line with data on various *Plasmodium* strains (2).

Furthermore, the above-mentioned study from Schmidt and colleagues (28) demonstrated higher activity of desbutyl drugs against two *Plasmodium* species. A similar result was observed in the present study against schistosomes. This similarity between the two parasites with respect to structural needs might point to similar modes of action of arylmethanols against schistosomes and *Plasmodium* species.

Regarding the 4-quinolinemethanols, we observed high *in vitro* activity for three of four representatives (compounds 8, 9, and 13) and moderate (compound 8) to very good (compound 9, 12, and 13) *in vivo* activity for all four compounds. WR7930 (compound 9), which possesses a piperidyl substituent on the methanol side chain, showed the highest worm burden reduction, followed by its desbutyl and dibutyl relatives. A correlation between the side-chain modification (dibutyl, desbutyl, and piperidyl substituent) and antischistosomal activity was observed *in vitro* for all compounds examined on NTS. All compounds tested with dibutyl substituents showed greatly reduced activities, in contrast to the piperidyl derivatives, which revealed high activities. A similar trend was observed for activity against the adult worms. Interestingly, these structure-activity correlations were also described for 4-quinolinemethanols tested against *Plasmodium* (29). Despite the exceptionally good activity of WR7930, it has to be mentioned that the drug possesses mild to moderate phototoxic properties (27). This fact in particular has to be kept in mind, as the drug would be used in tropical surroundings.

Mefloquine and enpiroline demonstrated similar activities *in vivo* and *in vitro*, according to the results obtained with both IMC and microscopy. Both compounds immediately affected the motility of adult *S. mansoni*. In contrast, a delayed loss of motility and a reduction of heat flow after exposure to WR7930 were observed using IMC. On the other hand, the highest worm burden reductions *in vivo* were observed with WR7930. The high *in vivo* activity of WR7930 might be explained by excellent pharmacokinetic properties. A long half-life of up to 25 to 30 days for the drug in humans was previously described (24).

In our work, we also studied the *threo* diastereomers of enpiroline. Earlier studies related to malaria research did not detect significant differences in the activities of *erythro* and *threo* diastereomers. Based on these data, the *erythro* isomers were not tested in the present work. An additional advantage of the *threo* isomers is that it is easier to prepare these compounds in larger quantities

TABLE 6 Activity of enpiroline, mefloquine, and WR7930 at doses of 100 and 200 mg/kg against *S. haematobium* in mice and hamsters[a]

Host	Drug [dose (mg/kg)]	No. of animals investigated	No. of animals cured	Mean no. of worms (SD) Liver	Mesenteric veins	Total	TWR (%)	*P* value
NMRI mice	Control	12		2.3 (2.4)	3.0 (4.0)	5.3 (5)		
	Enpiroline [200]	4	3	0 (0)	1.3 (2.5)	1.3 (2.5)	76.6	0.05
	Mefloquine [200]	4	1	0.7 (1.2)	0.3 (0.6)	1.0 (1.0)	81.3	0.03
	WR7930 [200]	4	4	0 (0)	0 (0)	0(0)	100.0	0.01
Syrian golden hamsters	Control	4		5.5 (4.2)	13.7 (6.7)	15.8 (4.6)		
	Mefloquine [100]	4	0	1.5 (1.9)	4.5 (2.6)	6.0 (2.9)	61.9	0.02
	Mefloquine [200]	4	3	0.3 (0.5)	0.8 (1.5)	1.0 (2.0)	93.7	0.02

[a] SD, standard deviation; TWR, total worm burden reduction; FWBR, female worm burden reduction.

(28). Our finding that both isomers and the racemate show similar antischistosomal activities is again in accordance with data on *P. falciparum* (11). A recent study by Manneck et al. showed that there was no significant difference between the activities of the diastereomers and its enantiomers of mefloquine in *S. mansoni*-infected mice (16). Interestingly, the (+)-*threo* isomer showed high toxicity at a dosage of 200 mg/kg in *S. mansoni*-infected mice (4 out of 5 treated mice died), while the (−)-*threo* isomer was well tolerated. Toxicity studies performed by the WRAIR in connection with preclinical trials for antimalarial candidates documented low levels of toxicity, with similar patterns determined for the two enantiomers (unpublished findings). Details on *in vitro* cytotoxicity assessments performed using two different cell lines are provided in Tables S1 and S2 in the supplemental material. Previous studies have shown that *threo* enpiroline has acceptable levels of acute and subacute toxicity in mice (28). In addition, the drug was well tolerated in clinical trials using total dosages of 1,500 mg (7). Nonetheless, our observations should be kept in mind, as the (−)-*threo* isomer might offer a therapeutic benefit in terms of minimized adverse events.

Though the activity of mefloquine against *S. mansoni* and *S. japonicum* in rodents has been well described (13, 37), we have for the first time elucidated the efficacy of mefloquine against *S. haematobium* in mice and hamsters. *S. haematobium* represents one of the most important human-pathogenic *Schistosoma* species (26). It is therefore of high importance to find a potential drug candidate which shows activity against all major *Schistosoma* species. Promisingly, no species-specific sensitivity was observed with all three lead candidates (mefloquine, enpiroline, and WR7930), revealing comparable activities against *S. mansoni* and *S. haematobium*. However, since the mouse is a poor host for drug testing against *S. haematobium* (as evidenced by the very low worm recoveries in mice), the results obtained for enpiroline and WR7930 should be confirmed in hamsters (as was done for mefloquine), since it is the better host for rodent *S. haematobium* infections (1, 5).

The degradation of hemoglobin provides adult schistosomes with essential nutrients such as amino acids for growth, development, and reproduction (31). Aggregation into hemozoin represents a heme (the resulting end product of hemoglobin digestion) detoxification pathway in *S. mansoni* (22). Mungthin and colleagues showed that hemoglobin degradation plays a central role in the mechanism of 4-aminoquinolines on *Plasmodium* species (20), and drug hematin binding was suggested as a possible mechanism (10). Another study postulated that exposure of *S. mansoni* worms to chloroquine inhibits heme aggregation (21). Our *in vitro* studies have demonstrated that the addition of hemoglobin to the incubation medium strongly impacts the activity of quinolinemethanols. Interestingly, pronounced activities in the presence of hemoglobin were detected only for representatives of the quinolinemethanols tested, WR 7930 and mefloquine. Enpiroline, a pyridinemethanol, did not show any differences in activity under different culture conditions. Surprisingly, the addition of red blood cells, which contain lots of hemoglobin, had no influence on the activity of the test drugs. One reason might be that an impact of red blood cells on activity would be visible only over a longer duration of incubation, since red blood cells have to be lysed in order to liberate intracellular hemoglobin. Overall, all the drugs (mefloquine, enpiroline, and WR7930) revealed good antischistosomal activity without addition of hemoglobin, hemin, or RBC, suggesting the presence of a second, non-heme-dependent mode of action for their antischistosomal activity. Similarly, an interaction with hematin polymerization may not be sufficient to explain the activity of such drugs as mefloquine against *Plasmodium* (20). We have recently demonstrated that mefloquine interferes with glycolysis in *S. mansoni* schistosomula (19).

In conclusion, our study has confirmed the high antischistosomal activity of compounds with an mefloquine scaffold. We identified four candidates, WR7930, its two derivatives, and enpiroline, that are characterized by high antischistosomal properties *in vivo*. Interestingly, two of these compounds have already undergone extensive testing as antimalarial drugs.

WR7930 showed the highest activity *in vivo*; however, due to its phototoxic potential, as observed in a previous study performed with humans (24), it does not represent a lead candidate. Nonetheless, both derivatives of WR7930 tested in the present work showed remarkably high antischistosomal activities; hence, these compounds should be studied in preclinical studies in greater detail. In addition, this chemical structure might serve as a starting point for further structure-activity studies. Finally, enpiroline offers the advantages that it has already been used in clinical trials and is characterized by a good pharmacokinetic and safety profile. Hence, the drug would be available for clinical testing in proof-of-concept studies. However, whether the drug offers a therapeutic benefit superior to that of mefloquine remains to be confirmed.

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3.2 Orally Active Antischistosomal Early Leads Identified from the Open Access Malaria Box

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Orally Active Antischistosomal Early Leads Identified from the Open Access Malaria Box

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Abstract

Background: Worldwide hundreds of millions of schistosomiasis patients rely on treatment with a single drug, praziquantel. Therapeutic limitations and the threat of praziquantel resistance underline the need to discover and develop next generation drugs.

Methodology: We studied the antischistosomal properties of the Medicines for Malaria Venture (MMV) malaria box containing 200 diverse drug-like and 200 probe-like compounds with confirmed *in vitro* activity against *Plasmodium falciparum*. Compounds were tested against schistosomula and adult *Schistosoma mansoni in vitro*. Based on *in vitro* performance, available pharmacokinetic profiles and toxicity data, selected compounds were investigated *in vivo*.

Principal Findings: Promising antischistosomal activity (IC_{50}: 1.4–9.5 µM) was observed for 34 compounds against schistosomula. Three compounds presented IC_{50} values between 0.8 and 1.3 µM against adult *S. mansoni*. Two promising early leads were identified, namely a N,N′-diarylurea and a 2,3-dianilinoquinoxaline. Treatment of *S. mansoni* infected mice with a single oral 400 mg/kg dose of these drugs resulted in significant worm burden reductions of 52.5% and 40.8%, respectively.

Conclusions/Significance: The two candidates identified by investigating the MMV malaria box are characterized by good pharmacokinetic profiles, low cytotoxic potential and easy chemistry and therefore offer an excellent starting point for antischistosomal drug discovery and development.

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Introduction

With hundreds of millions of people living at risk of infection and 207 million people infected with schistosomes worldwide, schistosomiasis is one of the most devastating parasitic diseases in tropical countries and remains a major public health problem, especially in Sub-Saharan Africa [1,2]. *Schistosoma haematobium*, *S. japonicum* and *S. mansoni* are the main schistosome species, responsible for the largest number of infections [3,4].

A major cornerstone of schistosomiasis control programs is the treatment of at risk populations with praziquantel, with the aim of controlling morbidity and preventing associated mortality [5–7]. Praziquantel, discovered in the 1970's, is the only drug available for the treatment of schistosomiasis [7–9].

Despite many benefits of praziquantel, most notably its high efficacy and excellent tolerability, the drug has major drawbacks, most importantly its inefficacy against juvenile schistosomes [10,11]. Furthermore the increasing administration of praziquantel to millions of people annually [12] results in high drug pressure, and thus drug-resistant parasites are likely to evolve [13].

These facts underline the urgent need to discover and develop the next generation of antischistosomals. Only a few compounds are presently being studied in the preclinical phase [14–17] and none of the candidates evaluated in clinical trials in the past years (e.g. mefloquine [18] or the artemisinins [19]) (Figure S1) met the target product profile for a novel antischistosomal drug [20].

Interestingly many of the chemical scaffolds that revealed promising activity against schistosomes had their origin in antimalarial research and discovery [21]. The blood-feeding characteristic that both parasites have in common forms the basis for the dual antimalarial and antischistosomal activity of drugs interfering with the parasites' hemoglobin degradation pathway [22,23].

The aim of the present study was to investigate the antischistosomal properties of the Medicines for Malaria Venture (MMV)

Author Summary

To date, praziquantel is the only available drug for the treatment of the tropical neglected disease schistosomiasis and is widely used in morbidity control programs. To discover new chemical scaffolds for the treatment of schistosomiasis, we investigated the Medicines for Malaria Venture malaria box containing 200 diverse drug-like and 200 probe-like compounds with known antimalarial activity against *Schistosoma mansoni*. Compounds were first investigated on the larval stage of *S. mansoni*, followed by testing against adult worms *in vitro* and by *in vivo* studies of lead candidates. We identified two entirely new chemical scaffolds: the N,N′-diarylurea and 2,3-dianilinoquinoxaline derivatives with antischistosomal *in vitro* activity in the sub micromolar range and significant activity in the mouse model. Since both compounds offer a good pharmacokinetic profile, low cytotoxic potential and easy chemistry, structure-activity relationship studies should be launched.

malaria box containing 200 diverse drug-like compounds (which fit in the "Lipinski space" or rule of five), as a starting point for oral drug discovery and development, and 200 diverse probe-like compounds (no filters applied). Note that all of the compounds in the box have confirmed activity against the blood-stage of *Plasmodium falciparum in vitro* and are commercially available [24]. Studying this diverse set of molecules might reveal an entirely new chemical scaffold for antischistosomal drug discovery and therefore fill up the empty antischistosomal drug pipeline.

At the Swiss Tropical and Public Health Institute (Swiss TPH), drugs were first studied against schistosomula *in vitro* followed by a re-evaluation of successful hits on adult *S. mansoni*. In parallel all the drugs were independently tested at the London School of Hygiene and Tropical Medicine (LSHTM) in an *in vitro* adult worm assay. Possible class effects and structure-activity relationships are discussed. The onset of action and IC_{50}/IC_{90} ratios were studied. Based on *in vitro* performance and available pharmacokinetic profiles as well as toxicity data, selected compounds were investigated *in vivo*.

Methods

Drugs and Media

The MMV Box [24], containing 400 compounds as stock solutions dissolved in dimethylsulfoxide (DMSO), concentration 10 mM, was kindly provided by MMV/SCYNEXIS, Inc. (Geneva, Switzerland; Durham, USA). For the *in vitro* studies on adult worms at the Swiss TPH and the *in vivo* studies in mice 5–100 mg of **1**: MMV000963, **2**: MMV665852, **3**: MMV665807, **4**: MMV019555, **5**: MMV019918, **6**: MMV000445, **7**: MMV019780, **8**: MMV665927, **9**: MMV665941, **10**: MMV000634, **11**: MMV665830, **12**: MMV666054, **13**: MMV009063, **14**: MMV007591, **15**: MMV665969, **16**: MMV666070, **17**: MMV007224, **18**: MMV665794, **19**: MMV666057, and **20**: MMV665799 were purchased from Specs (Delft, Netherlands), and MolPort (Riga, Latvia). Praziquantel was purchased from Sigma-Aldrich (Buchs, Switzerland) GmbH. Compounds **1–20** were dissolved in DMSO for drug stock solutions of 10 mg/ml for *in vitro* evaluations. Culture medium for newly transformed schistosomula (NTS) was made by supplementing Medium 199 (Lubioscience, Lucerne, Switzerland) with 5% heat-inactivated fetal calf serum (iFCS), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Lubioscience, Lucerne, Switzerland). Culture medium for adult worms was prepared by supplementing RPMI 1640 with 5% iFCS, penicillin (100 U/ml), and streptomycin (100 µg/ml).

Preparation of Newly Transformed Schistosomula (NTS)

S. mansoni cercariae (Liberian strain) were harvested from infected intermediate host snails (*Biomphalaria glabrata*) following in-house standard procedures. Collected cercariae were mechanically transformed to NTS as described previously [25,26]. The obtained NTS suspension was adjusted to a concentration of 100 NTS per 50 µl using supplemented Medium 199. NTS suspensions were incubated (37°C, 5% CO_2 in ambient air) for a minimum of 12 to 24 hours until usage to ensure completed conversion into schistosomula [27].

Ethics Statement

In vivo studies were conducted at the Swiss TPH, Basel, and approved by the veterinary authorities of the Canton Basel-Stadt (permit no. 2070) based on Swiss cantonal and national regulations. Experimentation at LSHTM was carried out under the UK Animals Scientific Procedures Act 1986 with approval from the LSHTM Ethics committee.

Maintenance of Mice and Infection with *S. mansoni*

Animals (female NMRI, 3-week old, weight ca. 14 g) were purchased from Charles River (Sulzfeld, Germany) and allowed to adapt under controlled conditions (temperature ca. 22°C; humidity ca. 50%; 12-hour light and dark cycle; free access to rodent diet and water) for one week. Mice were infected by subcutaneous injection with ~100 *S. mansoni* cercariae each, harvested from infected snails. For *in vitro* studies on adult flukes, schistosomes were collected from the hepatic portal and mesenteric veins of infected mice 7–8 weeks post infection [28]. Freshly harvested schistosomes were placed in supplemented RPMI culture medium, quickly rinsed, and stored at 37°C, 5% CO_2 until usage.

In Vitro Compound Screening Cascade on *S. mansoni* at Swiss TPH

Initially, all compounds were tested at a concentration of 100 µM on *S. mansoni* NTS. Active compounds progressed into a secondary screening at 33.3 µM. For this purpose drug stock solutions were diluted in 96-flat bottom well plates (BD Falcon, USA) with supplemented Medium 199 and 50 µl of prepared NTS suspension (100 NTS/well) to the desired final concentration of 100 µM or 33.3 µM, respectively. Each drug was tested at least in triplicate and the highest concentration of DMSO served as control. Plates were incubated at 37°C, 5% CO_2. NTS were evaluated by microscopic readout (Carl Zeiss, Germany, magnification 80–120×) using a viability scale as previously described with regard to death, changes in motility, viability, and morphological alterations 72 hours post drug exposure [25,26]. To ensure the accuracy of our assay, 45 compounds that lacked activity at one of the tested concentrations, were randomly selected and retested at 33.3 µM. Compounds that killed the NTS at 72 hours after exposure in at least one well were deemed active and selected for further testing.

In the next step, the IC_{50} was determined for active compounds from the preceding screens. Drug dilution series were prepared in 96-flat bottom well plates with concentrations 2.1, 4.2, 8.4, 16.7, and 33.3 µM using supplemented culture medium. The prepared NTS suspension was then added to each well and plates were incubated at 37°C, 5% CO_2. NTS incubated in the presence of the highest DMSO concentration and praziquantel served as control. Drug effects on NTS were evaluated 72 hours post exposure, using a viability scale, as described above. Each concentration was tested in duplicate and experiments were repeated once.

Compounds presenting IC_{50} values ≤10 μM were then tested at a concentration of 33.3 μM on adult worms in duplicate. Drug stock solutions (10 mM) were diluted in supplemented RPMI 1640 culture medium reaching a final concentration of 33.3 μM in 24-flat bottom well plates (BD Falcon, USA) within a final volume of 2.4 ml. At least three schistosomes of both sexes were added to each well. Schistosomes incubated in the presence of the highest concentration of DMSO served as control. Plates were incubated for 72 hours at 37°C, 5% CO_2. Seventy-two hours post drug exposure *S. mansoni* were examined phenotypically by microscope using the motility scale described before [29]. Drugs leading to the death of schistosomes 72 hours post exposure were characterized further and their IC_{50} (IC_{90}) values were determined. Specifically, drug dilution series were prepared in 24-flat bottom well plates (BD Falcon, USA) with concentrations of 0.31, 0.93, 2.78, 8.33, and 25.0 μg/ml using supplemented RPMI culture medium and freshly prepared drug stock solutions (10 mg/ml). At least three schistosomes of both sexes were added to each well and plates were incubated at 37°C, 5% CO_2. Parasites incubated in the highest DMSO concentration and praziquantel served as controls. Drug effects were evaluated 72 hours post exposure as described above. Each concentration was tested in duplicate and trials were repeated once.

In Vitro Screening on Adult Schistosomes at LSHTM

Adult worm drug testing was performed as previously reported [29] with some modifications as described. Worms of a Puerto Rican strain of *S. mansoni* were obtained by portal perfusion of CD1 mice (Charles River, UK) 6 weeks post-infection. Three pairs of worms were added to the wells of 48-well plates (Nunc, UK) in 1 ml complete DMEM medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (cDMEM). Compounds were tested at 15 μM containing 0.15% DMSO in single wells. Negative controls contained worms cultured in cDMEM alone and in cDMEM with 0.15% DMSO. Positive control wells contained worms cultured in praziquantel (Sigma-Aldrich, UK) at 10 μM. Cultures were incubated at 37°C and 5% CO_2. Effects were assessed on day 5 of culture using an inverted microscope (Leitz Diavert Wetzlar, Germany). Any compounds producing complete immotility or ≥70% worm motility inhibition plus severe morphological damage were considered hits in the primary screen [29]. Active compounds were then tested for IC_{50} value determination at a concentration range from 0.55–15 μM in single wells.

In Vitro Characterization of Lead Candidates on Adult Schistosomes

The onset of action (length of time needed before an antischistosomal effect was visible) was determined for selected compounds *in vitro* by evaluating the IC50 at a time-range of 1–72 hours (1, 2, 4, 7, 10, 24, 48, and 72 hours) post drug exposure, as described above. The onset of action of praziquantel was also studied. Additionally, possible protein binding effects were studied for three lead candidates and praziquantel. For that purpose RPMI medium was supplemented with two different iFCS concentrations (0% and 50%) and IC50 values were calculated for the different conditions. Furthermore, IC50 values were determined after varying drug exposure times (1, 2, or 4 hours) followed by incubation in drug free RPMI medium for 72 hours.

In Vivo Screening Using the Chronic *S. mansoni* Mouse Model

Groups of 3–4 NMRI mice characterized by a patent *S. mansoni* infection (49 days post-infection) were treated orally with the test drug using either single oral doses of 400 mg/kg or 80 mg/kg administered on four consecutive days. An additional dosage regimen of 100 mg/kg administered four times every 4 hours was tested for the 2 most active compounds (**2**, **17**). Compounds were freshly prepared in an aqueous hydroxypropyl methyl cellulose (HPMC) (1%): DMSO (95:5) formulation. Eight to sixteen untreated mice served as controls. Fourteen days post-treatment animals were killed by the CO_2 method and were dissected and the worms were sexed and counted [28]. Mean worm burdens of treated mice were compared to the mean worm burden of untreated animals and worm burden reductions were calculated.

Statistics

Parasite viability values of NTS and adult schistosomes obtained from microscopic evaluation were averaged (means (+/− standard deviation)) using Microsoft Excel. IC50 and IC90 values of test compounds were determined using the CompuSyn software (Version 3.0.1, 2007; ComboSyn Inc., USA) and Microsoft XLfit version 5.1.0.0 (2006–2008 ID Business Solutions Ltd). Selectivity indices were calculated by dividing the IC50 of the MRC-5 cells-fibroblast cytotoxicity data by the IC50 of the adult worm assay. The Kruskal-Wallis test was applied for *in vivo* studies, comparing the worm burden of the treated animals and control animal groups. A difference in worm burden was considered to be significant at a significance level of 5% (StatsDirect, version 2.7.2.; StatsDirect Ltd., UK).

Results

In Vitro Activity Determined on NTS and Adult Schistosomes at Swiss TPH

Exposing schistosomula to the test drugs (n = 400) at a concentration of 100 μM resulted in death of NTS for 45% of the tested compounds (n = 179). Schistosomicidal effects were observed for 18% of these active compounds (n = 72) at the lower concentration of 33.3 μM (Figure 1). A diverse range of chemical scaffolds was observed amongst active compounds. Successful candidates were characterized further on NTS. Promising antischistosomal activity (IC_{50}: 1.4–9.5 μM) was observed for 34 compounds, two of which were identified during our quality control re-evaluation of 45 compounds and nine of which showed comparable or increased activity (IC_{50}: 1.4–2.4 μM) to praziquantel (IC_{50}: 2.2 μM).

All hits (IC_{50}<10 μM) (n = 34) were next tested at a concentration of 33.3 μM on adult *S. mansoni.* Seventy-two hours post drug exposure, 16 (**1–16**) of these compounds (Table S1) killed the adult worms. Four of the ten compounds with high activities (IC_{50}<2.5 μM) on NTS lacked antischistosomal activity on adult worms. The 16 active candidates were further characterized by IC_{50} value determination. The highest *in vitro* activities were observed for the diaminoquinazoline derivative **1** (IC_{50}: 0.8 μM) the diarylurea **2** and diarylamide **3**, presenting IC_{50} values of 0.8 and 1.3 μM, respectively (PZQ: 0.2 μM). IC_{50} values ranging from 2.6–9.2 μM were calculated for compounds **4**–**11**, whereas only moderate activity (IC_{50} values >10 μM) was determined for five compounds (**12–16**). Compounds with IC_{50}>10 μM were excluded from further consideration, meaning only eleven compounds were considered as hits.

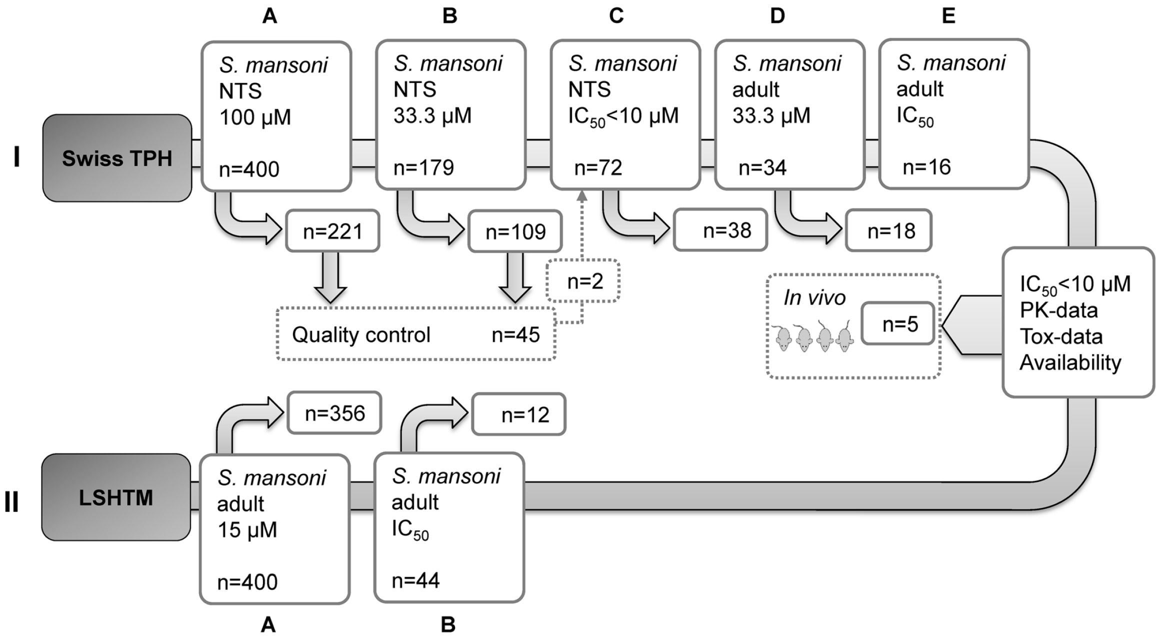

Figure 1. Screening flow. Screening was conducted at the Swiss TPH (screening cascade **I**; steps **A**–**E** and Quality control): Primary screening steps (yes/no filters) of 100 µM and 33.3 µM resulted in 179 and 72 hits, respectively. Active compounds (n = 72) moved on to Step **C** and IC_{50} values were evaluated on NTS. Thirty-four compounds showed activities with IC_{50} values <10 µM and pre-screening was conducted on adult schistosomes (Step **D**). Active compounds (n = 16) with schistosomicidal effects at 33 µM compound concentration were further characterized (step **E**). The quality control represents randomly selected compounds from compounds classified as non-active from the pre-screening steps (step **A**/**B**) on NTS which were re-evaluated in step **B**. In parallel, all compounds (n = 400) were studied at the LSHTM in London (screening cascade **II**): step **A**, all 400 compounds were screened on *S. mansoni* adults at 15 µM. Step **B: 44** compounds were active and these were then tested for IC_{50} determination on adult worms. From both screening cascades, five compounds were selected for *in vivo* testing based on pharmacodynamic and pharmacokinetic properties as well as toxicity.
doi:10.1371/journal.pntd.0002610.g001

In Vitro Activity Determined on Adult Schistosomes at LSHTM

Forty-four compounds were classified as hits (compounds producing complete immotility or ≥70% worm motility inhibition plus severe morphological damage) against adult *S. mansoni in vitro* at a concentration of 15 µM. These compounds were further tested for IC_{50} values (Table S1). Twelve compounds showed IC_{50} values >15 µM. Fourteen compounds revealed IC_{50} values between 10–15 µM. Eighteen compounds had IC_{50} values <10 µM. To provide a comparison with the Swiss TPH assays, the 32 hits were subsequently tested using the schistosomula assay at LSHTM [30]. This showed generally good concordance with the LSHTM adult assay, in that all adult hits with IC_{50}<10 µM were also hits in the larval assay (Table S1).

Selection of Lead Candidates

Based on *in vitro* performance on the adult worms (Table S1), toxicity, pharmacokinetic (PK) properties and availability of the compounds, five lead candidates (**1**, **2**, **5**, **8**, **17**) (Figure 2) were selected for *in vivo* testing and in depth characterization *in vitro*. In more detail, 11 compounds were excluded after comparing their IC_{50} values and PK parameters (C_{max}, t_{max}, $t_{1/2}$, AUC). Four compounds showed poor antischistosomal activity (IC_{50}>10 µM) and four compounds showed poor bioavailability (C_{max}<IC_{50} of the corresponding compound). Ten compounds were characterized by low selectivity indices (SI<1) and two were not commercially available.

Four active compounds were derivatives belonging to the class of diarylureas and two compounds were characterized as dianilinoquinoxalines. Only the most active candidate of each chemical group, compound **2** and compound **17**, was selected for *in vivo* studies. A summary of the IC_{50} values, toxicity and pharmacokinetic parameters of the lead candidates is provided in Table 1.

In Vitro Characterization of Lead Candidates on Adult Schistosomes

The onset of action was studied in compounds selected for *in vivo* testing (n = 5) and compared to the onset of action for praziquantel (Figure 3). Compound **2** was the fastest acting drug, presenting an IC_{50}<5 µM already after 1 hour of *in vitro* exposure, followed by compound **17** with an IC_{50}<10 µM, 1 hour post incubation. Compound **1** was intermediate in speed with an onset time of 7 hours post-incubation. Compound **8** had fully exerted its antischistosomal properties 24 hours following incubation, while compound **5** was slow acting (exposing its full antischistosomal activity only 72 hours post treatment). In comparison, praziquantel exposed its entire antischistosomal activity already after 1 hour of drug exposure (IC_{50}: 0.2 µM).

The determined IC_{90} values of the lead candidates were 2–5 fold higher than the observed IC_{50} values 72 hours post exposure and thus the concentration-response curves for these compounds are quite steep (Table 2). Comparatively, praziquantel even showed a 13-fold difference between the two values. Praziquantel

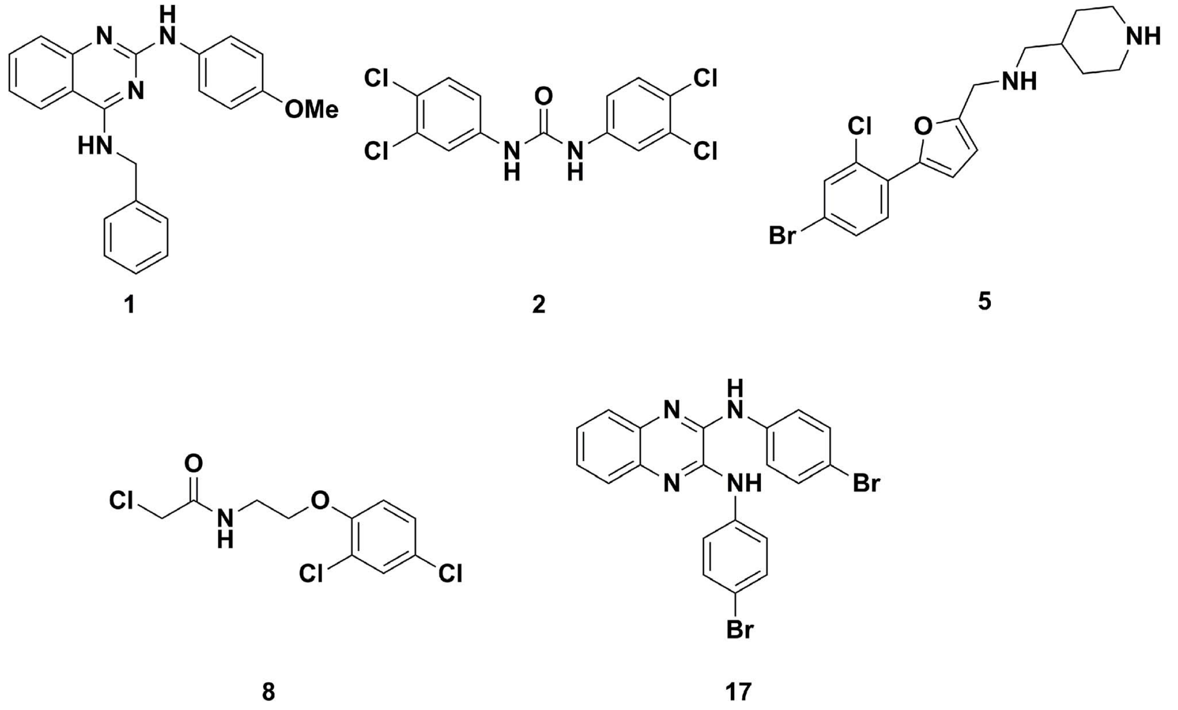

Figure 2. Chemical structures of the five lead compounds selected for *in vivo* studies.
doi:10.1371/journal.pntd.0002610.g002

lead very quickly to a strong motility inhibition and morphological changes, whereas higher concentrations (IC$_{90}$: 2.0 µM) were necessary to actually kill the worms.

In Vivo Findings

Compound **2** and **17** revealed the highest *in vivo* activity with worm burden reductions (WBR) of 52.5% (dosage 1×400 mg/kg; $p<0.005$) and 53.4% (dosage 4×100 mg/kg; $p<0.005$), respectively (Table 3). In addition, both treatment regimens using multiple doses of compound **2** resulted in significant worm burden reductions of 46.0% (4×80 mg/kg; $p<0.005$) and 31.2% (4×100 mg/kg; $p<0.05$). Treatment with a single 400 mg/kg dose of compound **17** resulted in a significant worm burden reduction of 40.8% ($p<0.05$), while multiple treatment courses of 80 mg/kg over four consecutive days achieved a lower effect (WBR: 25.5%, $p<0.05$). Compounds **1**, **5**, and **8** lacked *in vivo* activity (WBR 0–18.7%). No significant differences were observed between total and female worm burden reductions.

Protein-Binding and Short-Term Drug Exposure of Leads

Compound **17** showed a 7-fold increase in activity in iFCS-free medium (IC$_{50}$: 0.3 µM) versus incubation in 50% serum supplemented medium (IC$_{50}$: 2.1 µM) (Table S2). A strong increase in activity in serum free medium was observed for praziquantel (IC$_{50}$: 0.02 µM). No altered activities were detected for compound **2** within varying iFCS-concentrations. Short-term exposure of schistosomes to compound **2** or praziquantel (1–4 hours) followed by incubation in drug free medium for 72 hours resulted in high IC$_{50}$ values, ranging from 51.1 µM (1 hour) to 24.6 µM (4 hours) for compound **2** and from 96.1 µM to 7.7 µM for praziquantel (Figure S3). These values are much higher than the IC$_{50}$ values determined when the worms are continuously exposed to the drugs for 72 hours (**2**: IC$_{50}$: 0.8 µM; PZQ: IC$_{50}$: 0.2 µM). Incubation of schistosomes for 4 hours with compound **17** achieved similar effects (IC$_{50}$: 1.3 µM) (Figure S3) as described for the 72 hours exposure time (IC$_{50}$: 0.8 µM) (Table S2).

Discussion

The aim of this study was to investigate the antischistosomal potential of 200 drug-like and 200 probe-like compounds assembled in the MMV Malaria Box. The MMV Malaria Box provided a unique opportunity: commercially available compounds with confirmed *in vitro* activity against *P. falciparum* serve as good starting material for antischistosomal R&D, as many antimalarials have antischistosomal activity [16,23,31]. In addition, and in line with the target characteristics of a trematocidal lead candidate [20], properties of the drug-like compounds are commensurate with oral absorption and the presence of known toxicophores is minimized.

NTS were used as a prescreening tool at Swiss TPH, since their use greatly reduces the need for laboratory animals and thus is a major contributor to the 3 R rules (replace, reduce, refine) [25]. Nearly half of the tested compounds (45%) presented schistosomicidal effects on the schistosomular stage at a concentration of 100 µM. Given this high hit rate, compounds which were not lethal on NTS did not progress further. This might be a limitation of the Swiss TPH screening, since many effective anthelmintics (including praziquantel at low concentrations) cause paralysis rather than death of worms [32]. Thirty-four of the active compounds had IC$_{50}$ values ranging from 1.4 to 9.5 µM, suggesting that both parasites, *P. falciparum* and *S. mansoni*, have a similar drug sensitivity profile. About half of the compounds active against NTS ($n=16$) revealed good to moderate activity on

Table 1. Characterization of five lead candidates selected for *in vivo* testing.

			P. falciparum 3D7		NTS		Adult *S. mansoni*		MRC-5 cells-fibroblast		PK- data				
Compound	Molecular weight (g/mol)	ALogP	Inhibition at 5 μM (%)	EC_{50} (nM)	IC_{50} (μM)	R	IC_{50} (μM)	R	IC_{50} (μM)	Selectivity index	Dosage (mg/kg)	C_{max} (μmol/l)	t_{max} (hours)	$AUC_{0\text{-last}}$ (h*μM/l)	$t_{1/2}$ (hours)
1	356.42	4.3	-	589	2.7	0.9	0.8	0.9	12.38	15.48	47.8	0.054	7	0.37	>>3
2	350.03	5.2	96	1160	4.7	1	0.8	1	32.00	40.00	46.3	4.4	4.7	30.2	NR
5	383.71	3.9	96	800	1.8	0.9	3.4	0.9	4.03	1.18	50.3	0.37	1.1	1.7	5.2
8	282.55	2.9	94	555	3.4	1	6.3	0.9	16.23	2.58	40.3	0.57	0.3	0.9	2.4
17	470.16	5.7	98	1061	-	-	0.8	-	5.88	7.08	62.5	12.4	8	73	NR

In vitro activity on *P. falciparum* 3D7, NTS, adult *S. mansoni*, cytotoxicity on MRC5-cells, and pharmacokinetic parameters* of 5 active compounds selected for *in vivo* studies identified in 2 parallel screens at the Swiss TPH and LSHTM.

*PK parameters are unpublished data, *In vitro* activity on *P. falciparum* 3D7 and cytotoxicity on MRC5-cells can be found at http://www.mmv.org/research-development/malaria-box-results.

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the adult stage (IC_{50}: 0.8–22.3 μM). Several compounds that showed high antischistosomal effects on schistosomula lacked activity on adults. This phenomenon, where the hit rates were higher against the larval stages than against the adult stages, has been previously reported [14,33]. A higher sensitivity of the larval stage, or mode of action dependent effects might partially explain this higher hit rate: for example recent studies with various peroxide classes documented less activity on the adult stage than on the NTS stage [15].

The parallel screening at LSHTM screened all compounds directly on adult schistosomes. Thirteen additional compounds active against adult worms ($IC_{50}<10$ μM) were identified at LSHTM. Nine of these lacked activity against NTS at Swiss TPH (Figure S2). Interestingly, these compounds showed activity against NTS at LSHTM (Table S1). On the other hand, four compounds with activity ($IC_{50}<10$ μM) against NTS and adult worms identified at Swiss TPH lacked activity in the LSHTM screen. Overall, 22 compounds had an $IC_{50}<10$ μM against adult worms in at least one of the screens. Only five compounds were characterized by an $IC_{50}<10$ μM in both screenings. Strain differences but also different ways of assay set up and readout might offer an explanation for these results. Nonetheless, follow up studies to clarify these issues are warranted.

Compound **2**, a diarylurea, revealed the highest activity against adult *S. mansoni in vitro*. In addition, our onset of action studies revealed that it was the fastest acting compound, comparable to praziquantel. The compound is characterized by an intriguingly simple chemistry and can be easily synthesized. The class of N,N′-diarylureas was recently found to activate heme-regulated inhibitor kinase which inhibits translation initiation and plays a central role in cancer initiation [34]. Additionally various N,N′-diarylureas, including compound **2**, have been investigated as potential anti-cancer agents and were proposed as promising lead compounds [35]. Significant worm burden reductions of 52.5%, 46.0%, and 31.2% were observed with compound **2** following single oral dosing with 400 mg/kg, 80 mg/kg on four consecutive days and 4×100 mg/kg every four hours, respectively. This might indicate that *in vivo* activity follows a time over threshold model rather than it being C_{max} driven. However, based on the *in vitro* performance and pharmacokinetic data, a better *in vivo* outcome was expected. Our follow-up *in vitro* studies, which studied protein binding and the short-term drug exposure, might offer an explanation for this discrepancy. Short incubation times (1 to 4 hours) were not sufficient to kill the worms, since most of the parasites recovered 3 days later. Note that compound **2** is characterized by a half-life ($t_{1/2}$) of 4.7 hours and C_{max} of 4.4 μM at 46.3 mg/kg (*po*).

Additionally compound **17**, a 2,3-dianilinoquinoxaline derivative, showed high *in vitro* (IC_{50}: 0.83 μM) and significant *in vivo* activity with WBRs between 53.4% (multiple *po* dose of 100 mg/kg every four hours) and 40.8% (single *po* dose 400 mg/kg). This series has been reported to show antimycobacterial activity [36].

The order of *in vivo* activity of the five selected candidates is in line with the onset of action observed *in vitro*. The fastest acting compound **2** exhibited the highest activity *in vivo* followed by compound **17** (WBR: 40.8%). The discrepancy of excellent *in vitro* performance of compound **17**, but only moderate *in vivo* activity might be explained by protein binding effects. Increased activities were observed when incubated sans serum proteins *in vitro*. Notably, short-term incubation of 4 hours was sufficient to exhibit high antischistosomal effects for both drugs. Compounds **1**, **5**, and **8** acted slower (only 7–10 hours post exposure), and lacked activity *in vivo*. This finding is in line with PK properties of these drugs. Since the half-lives of the compounds are rather short

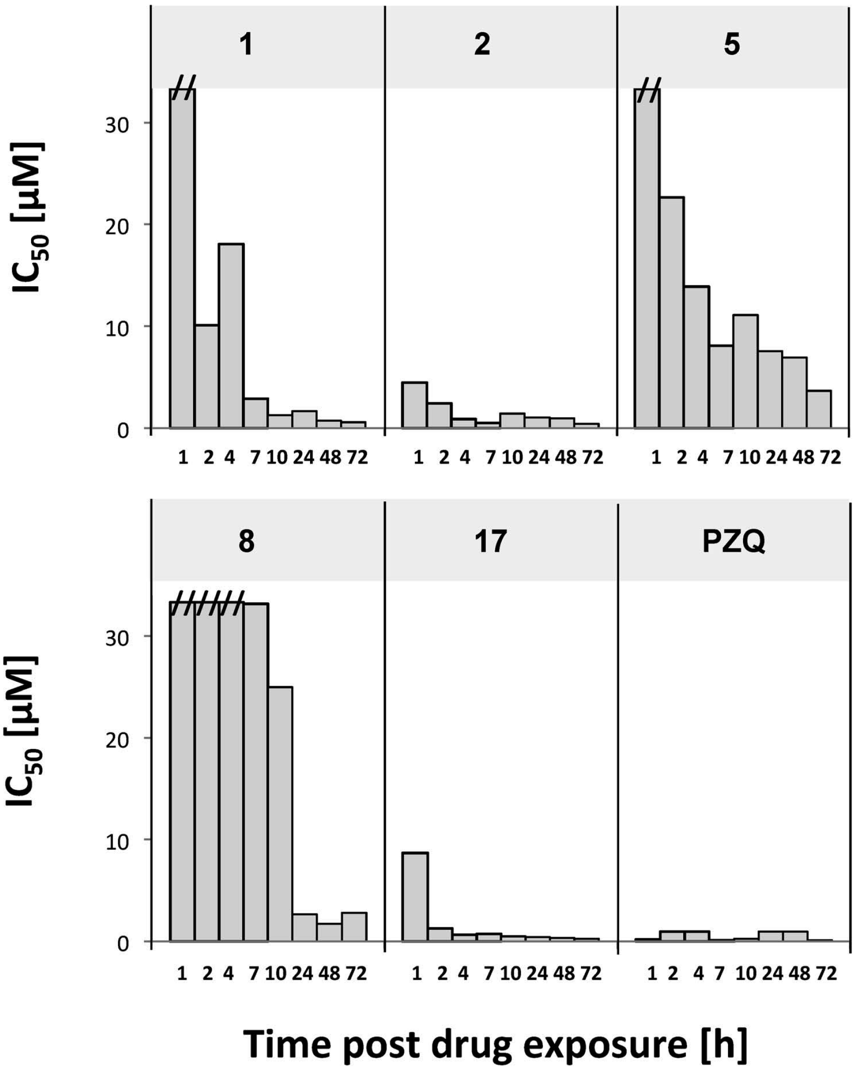

Figure 3. Adult worm IC$_{50}$ values of five *in vivo* candidates over time post drug exposure. Values were determined 1, 2, 4, 7, 10, 24, 48 and 72 hours after drug exposure. Values >33.3 µM are indicated with (//). PZQ: praziquantel.
doi:10.1371/journal.pntd.0002610.g003

Table 2. Adult worm IC$_{50}$ and IC$_{90}$ values of five *in vivo* candidates compared to praziquantel 72 hours post drug exposure.

Compound	1	2	5	8	17	PZQ
IC$_{50}$ (µM)	0.6	0.5	3.7	2.8	0.3	0.2
IC$_{90}$ (µM)	1.7	2.2	9.9	7.9	1.2	2.0
Ratio IC$_{90}$/IC$_{50}$	2.8	4.8	2.7	2.8	4.1	13.1

PZQ: Praziquantel.
doi:10.1371/journal.pntd.0002610.t002

(2.4–5.2 hours) plasma concentrations remain insufficiently long above the IC$_{50}$ values for the slow acting compounds to exert *in vivo* activity.

Since a series of related derivatives was present in the MMV Malaria Box, we carried out an initial structure-activity relationship study by sourcing commercially available near neighbors for compounds **1**, **2**, and **5** (Table S3). Exchanging the phenyl-group of **1** with an ethanol group revealed a stage specific sensitivity with activity on NTS, but lacked schistosomicidal effects on adult worms. The substitution pattern on phenyl-residues of compound **2** influenced activity. For example, exchanging the *para*-chloro to a *para*-fluro on one of the phenyl rings led to a two-fold decrease in activity on NTS. Such subtle changes in activity require further investigation with a larger set given the easy chemical accessibility of derivatives.

Table 3. Worm burden reductions observed for the five lead candidates in *S. mansoni* infected mice.

Compound	Dosage	Mice(n)	Total worms recovered (n)	SD	WBR (%)	Control batch
Control 1	-	16	38.5	13.2	-	-
Control 2	-	9	40.4	13.5	-	-
Control 3	-	8	35.4	13.8	-	-
1	1×400 mg/kg	4	50.3	12.7	0	1
	4×80 mg/kg	3	34.7	14.2	9.9	1
2	1×400 mg/kg	4	18.3	5.1	52.5**	1
	4×80 mg/kg	4	20.8	6.1	46.0**	1
	4×100 mg/kg	4	27.8	7.0	31.2*	2
5	1×400 mg/kg	4	37.8	8.1	1.8	1
	4×80 mg/kg	4	33.5	16.9	12.7	1
8	1×400 mg/kg	3	31.3	6.5	18.7	1
	4×80 mg/kg	3	31.7	8.5	17.7	1
17	1×400 mg/kg	4	22.8	10.9	40.8*	1
	4×80 mg/kg	3	28.7	10.1	25.5*	1
	4×100 mg/kg	4	16.5	8.5	53.4**	3

Mice harbored a patent *S. mansoni* infection. Different dosage regimens were used (1×400 mg/kg, 4×80 mg/kg on four consecutive days or 4×100 mg/kg every 4 hours).
WBR: Worm burden reduction.
*p-value <0.05.
**p-value <0.005.
doi:10.1371/journal.pntd.0002610.t003

In conclusion, by screening the MMV malaria box on *S. mansoni* we underlined the potential of compounds with an antimalarial background on schistosomes. We identified two entirely new chemical scaffolds: the N,N′-diarylurea (**2**) and 2,3-dianilinoquinoxaline derivatives (**17**) with antischistosomal *in vitro* activity in the sub micromolar range and moderate *in vivo* activity. The compounds offer promising drug characteristics such as a good pharmacokinetic profile and low cytotoxic potential. Their easy chemistry simplifies further drug optimization steps and offers an excellent starting point for antischistosomal drug discovery and development.

Supporting Information

Figure S1 Structures of anthelmintic and antimalarial drugs used against schistosomiasis.
(PPT)

Figure S2 Venn diagram for adult *S. mansoni* hits direct screening on adult schistosomes shown in blue (at LSHTM) or with prior screening on NTS followed by screening on the adult stage presented in red (at Swiss TPH).
(PPT)

Figure S3 Adult worm IC$_{50}$ values of the two lead candidates (**2** and **17**) incubated with the compounds for 1, 2, or 4 hours followed by incubation in compound-free medium for 72 hours.
(PPT)

Table S1 Results for the LSHTM and Swiss TPH *in vitro* adult and larval *S. mansoni* screening.
(DOC)

Table S2 IC$_{50}$ values of compounds **2**, **17** and praziquantel (**PZQ**) in RPMI medium supplemented with 0, 5, or 50% iFCS.
(DOC)

Table S3 *In vitro* performance of selected derivatives of *in vivo* candidates **1**, **2**, and **5**.
(DOC)

Author Contributions

Conceived and designed the experiments: JK KIS NRM QDB TS. Performed the experiments: KIS NC GP MV NRM QDB. Analyzed the data: KIS NC JK TS NRM QDB. Contributed reagents/materials/analysis tools: TS TNCW. Wrote the paper: KIS NC GP NRM QDB TNCW TS JK.

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Chapter 4

Disposition of mefloquine and enpiroline is highly influenced by a chronic *Schistososma mansoni* infection

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Disposition of Mefloquine and Enpiroline Is Highly Influenced by a Chronic *Schistosoma mansoni* Infection

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Chronic *Schistosoma mansoni* infections lead to severe tissue destruction of the gut wall and liver and can influence drug disposition. This study aimed to investigate the impact of a chronic *S. mansoni* infection on the pharmacokinetic (PK) parameters of two promising antischistosomal lead candidates (mefloquine and enpiroline) in mice. Studies were conducted in two different mouse cohorts (*S. mansoni*-infected and uninfected mice) for both drugs. Plasma samples were collected at various time points after oral treatment (200 mg/kg of body weight) with study drugs. A high-performance liquid chromatography (HPLC) method was validated to analyze enpiroline and mefloquine in plasma. Livers and intestines were collected from infected animals to determine the onset of action, hepatic shift, and worm burden reduction. Following mefloquine administration, hepatic shifting and significant worm burden reductions (79.2%) were observed after 72 h. At 1 week posttreatment with enpiroline, the majority of worms had migrated to the liver and significant worm burden reductions were observed (93.1%). The HPLC method was selective, accurate (87.8 to 111.4%), and precise (<10%) for the analysis of both drugs in plasma samples. The PK profiles revealed increased values for half-life ($t_{1/2}$) and area under the concentration-time curve (AUC) for both drugs in infected animals compared to the $t_{1/2}$ and AUC values in uninfected animals. Considerable changes were observed for mefloquine, with a 5-fold increase of $t_{1/2}$ (182.7 h versus 33.6 h) and 2-fold increase of AUC (1,116,517.8 ng · h/ml versus 522,409.1 ng · h/ml). *S. mansoni* infections in mice influence the PK profiles of enpiroline and mefloquine, leading to delayed clearance. Our data confirm that drug disposition should be carefully studied in schistosomiasis patients.

Schistosomiasis is one of the most important neglected tropical diseases (1). Based on the latest estimates, 780 million people are at risk of an infection with schistosomes, with the majority of cases in sub-Saharan Africa (2). Schistosomiasis is a chronic disease caused by the immunological response to eggs trapped in tissue and organs. For example, in the case of *Schistosoma mansoni*, eggs get trapped mainly in the gut wall and liver, leading to severe tissue destruction, hepatosplenomegaly, periportal liver fibrosis, and portal hypertension (3). Treatment with praziquantel is the mainstay of control and widely used in preventive chemotherapy programs (4).

The liver is one of the main organs in charge of drug metabolism, and the gut wall is significantly involved in the absorption process of drugs (5). It is therefore not surprising that pharmacokinetic (PK) changes occur in patients with schistosomiasis, depending on the extent of disease (6). For example, a study in Egyptian schistosomiasis patients treated with praziquantel demonstrated that PK parameters increased in proportion to the degree of hepatic insufficiency (7). In addition, PK studies undertaken with praziquantel in *S. mansoni*-infected mice documented elevated plasma levels in infected animals compared to the plasma levels in noninfected mice. Furthermore, the infection resulted in significant inhibition of microsomal cytochrome P450 activities compared with those in noninfected mice (8).

The areas of endemicity of schistosomiasis and some other infectious diseases, such as malaria and soil-transmitted helminths, overlap geographically (9). Coendemicity, as well as known similarities of both parasites, such as hemoglobin digestion (10, 11), were the key rationales for in-depth studies on the potential antischistosomal effects of antimalarials (9). In recent years, the antimalarial mefloquine emerged as a promising lead candidate. The drug presented remarkable *in vitro* and *in vivo* activities against major schistosome species (12) and high egg reduction rates in combination with artesunate in an exploratory clinical trial in *Schistosoma haematobium*-infected school-aged children (13). Recently, a clinical trial in pregnant malaria-infected women revealed a positive effect of mefloquine on *S. haematobium* coinfections (14). A population PK study of mefloquine observed altered PK profiles in malaria-infected persons compared to the PK profiles in uninfected people (P. Olliaro, personal communication). Nevertheless, the impact of a schistosome infection on the drug disposition of mefloquine has not been studied to date.

The aim of the present study was to investigate the PK parameters of mefloquine and enpiroline in the chronic *S. mansoni* infection mouse model. Enpiroline was included in this study since recent *in vitro* and *in vivo* studies on mefloquine-related arylmethanols identified enpiroline as a promising lead candidate with excellent activity on both *S. mansoni* and *S. haematobium* (12). Drug plasma levels were analyzed between 1 and 168 h after oral treatment with mefloquine and enpiroline. For that purpose, we adapted a previously established high-performance liquid chromatography (HPLC)-UV method (15), revalidated the method for mouse plasma samples, and expanded it for the quan-

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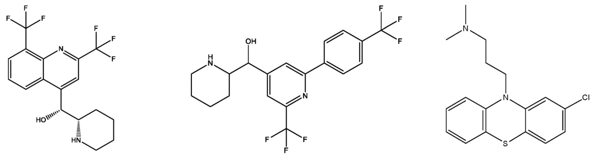

Mefloquine Enpiroline Chlorpromazine

FIG 1 Chemical structures of mefloquine, enpiroline, and chlorpromazine (internal standard).

tification of enpiroline. Finally, we determined the onset of action of both drugs following administration to infected animals.

MATERIALS AND METHODS

Drugs and chemicals. Mefloquine {[2,8-bis(trifluoromethyl)quinolin-4-yl](-piperidin-2-yl)methanol} was kindly provided by Cilag AG (Switzerland). Two mefloquine metabolites, one a human metabolite (Ro 21-5104) and one an animal metabolite (Ro 14-0518), were gifts of Hoffmann-La Roche (Switzerland). The Walter Reed Army Institute of Research (Silver Spring, MD) kindly provided us with enpiroline [threo-α-(2-piperidyl)-2-trifluoromethyl-6-(4-trifluoromethylphenyl)-4-pyridinemethanol]. Chlorpromazine [2-chlor-10-(3-dimethylaminopropyl) phenothiazin] was purchased from Sigma-Aldrich (Switzerland). Mefloquine and enpiroline were dissolved in dimethyl sulfoxide (DMSO) and chlorpromazine in ethanol (10 mg/ml stock solutions). All analytes are depicted in Fig. 1. Methanol (Sigma-Aldrich) and acetonitrile (Biosolve BV, Netherlands) were purchased in HPLC grade. Monobasic potassium phosphate and phosphoric acid (85%) were acquired from Sigma-Aldrich (Switzerland).

Animals and parasites. Animal studies were carried out at the Swiss Tropical and Public Health Institute (Basel, Switzerland) under protocols approved by Swiss national and cantonal animal welfare regulations. Three-week-old (weight, 14 g) female NMRI mice ($n = 119$) (Charles River, Sulzfeld, Germany) were allowed to adapt in the animal facilities for 1 week under controlled conditions (temperature, ca. 22°C; humidity, ca. 50%; 12-h-light and 12-h-dark cycle; and free access to rodent diet and water) before infection. Half of the mice (infected cohorts) were subcutaneously infected with 100 *S. mansoni* (Liberian strain) cercariae by following the standard procedures of our laboratory. Animals were then left under controlled conditions for 7 weeks to establish an early-stage chronic schistosome infection with visible impairment of liver (granulomatous tissue) and gut (swelling, inflammation). The remainder of the mice (uninfected cohorts) were likewise kept under controlled conditions for 7 weeks.

Pharmacokinetic studies. Pharmacokinetic studies were conducted in two different mouse cohorts for both drugs, one cohort being *S. mansoni*-infected and the second being noninfected NMRI mice. Oral formulations of enpiroline and mefloquine were prepared 3 h before treatment as water-based suspensions in 7% (vol/vol) Tween 80 and 3% (vol/vol) ethanol. Groups of mice ($n = 3$ per group) were treated orally with 200 mg per kg of body weight of enpiroline or mefloquine by gavage and sacrificed by the CO_2 method at selected time points posttreatment (1, 2, 4, 8, 12, 24, 48, 72, or 168 h). Whole-blood samples of 0.5 to 1 ml were collected by cardiac puncture of each mouse. Each cohort had untreated control mice ($n = 8$ infected and $n = 3$ noninfected), and each of the four treatment arms (infected and noninfected mice treated with mefloquine and infected and noninfected mice treated with enpiroline) consisted of 27 mice for 3 mice per sampling time point. Blood samples were collected into lithium-heparin-coated Microtainers (Sarstedt) and centrifuged to obtain plasma samples, which were stored at −80°C until analysis.

Studies on the onset of antischistosomal action in mice. The onset of action was determined at different time points following treatment with single oral doses of enpiroline and mefloquine administered to mice ($n = 3$ per group). After each PK sampling point (1, 2, 4, 8, 12, 24, 48, 72, or 168 h posttreatment), the liver and gut, including the portal vein and mesenterial veins, were dissected from infected mice. Livers were pressed and examined under the microscope, and all worms were sexed and counted (16). In addition, worms within the portal vein and mesenterial vein system were picked and counted separately as described previously (16). The worm burdens (average numbers of live worms) of treated mice were compared to the worm burdens of control (nontreated) animals (average numbers of live worms), and the reductions of worm burden (reduction of live worms) calculated for each time point. Furthermore, the onset of action, visible as the migration of worms from the mesenteric veins to the liver, was determined based on the distribution of worms (live and dead) within portal and mesenterial veins and pressed livers (hepatic shift).

HPLC-UV analysis and sample extraction. A validated high-performance liquid chromatography analytic method introduced by Lai and colleagues (15) for mefloquine in human plasma was used. The method was adapted in order to detect mefloquine and enpiroline simultaneously under similar conditions. Important validation parameters, such as selectivity, intra- and interday accuracy and precision, bench-top stability, and extraction recovery were determined according to the bioanalytical method validation guidance for industry of the Food and Drug Administration (FDA) (17).

Plasma samples (100 μl) were processed using protein precipitation with methanol containing an internal standard (IS) (chlorpromazine at a concentration of 5 μg/ml). Each mouse sample was extracted three times on three different days. Samples were vortex mixed and centrifuged (Eppendorf 5415C centrifuge) at 10,000 × *g* for 10 min. The supernatant was transferred to a microtube and evaporated to dryness in a Speedvac (Labcanco) at 38°C for 2 h. The residue was reconstituted in 100 μl acetonitrile–0.05 M KH_2PO_4 buffer (1:1) solution and transferred to an autosampler vial.

Analyte working solutions were prepared by serial dilution in acetonitrile-KH_2PO_4 buffer (0.05 M) (1:1) to final concentrations of 4, 8, 20, 40, 80, 200, 400, and 800 μg/ml. Calibration curves were established by diluting analyte working solutions with blank mouse plasma (purchased from Dunn Technik, Germany) (1:20 in a total volume of 100 μl). Each calibration set consisted of one blank plasma sample (plasma sample processed without IS), one zero sample (plasma sample spiked with IS), and 8 calibration samples (0.2, 0.4, 1, 2, 4, 10, 20, and 40 μg/ml). The lowest and highest calibrator corresponded to the lower and upper limit of quantification (LLOQ and ULOQ), respectively. The LLOQ was selected as the minimal concentration in plasma samples that could be analyzed with a precision of ≤20% (CV%) and accuracy of between 80% and 120% (signal/noise ratio of >5:1).

In addition, quality control (QC) samples ($n = 6$) were prepared at low (0.75 μg/ml), medium (7.5 μg/ml), and high (30 μg/ml) concentrations covering the entire calibration range.

Selectivity was determined by examination of blank mouse plasma obtained from different origins (noninfected NMRI mice and infected mice without treatment [$n = 6$ each] and commercially acquired mouse plasma) for interference by endogenous substances using the above-described extraction procedure but without adding the IS working solution.

The accuracy and precision of the method were evaluated by analyzing quality control (QC) samples ($n = 6$ per concentration). The intra- and interday accuracy/precision were determined within a single run and between different assays ($n = 3$), respectively. Freshly prepared calibration standards were used for the analyses. The precision was calculated using the coefficient of variation (CV [%]). The accuracy represented the measured concentration as the fraction of the nominal concentration expressed as a percentage. A precision of ±15% (LLOQ, ±20%) and accuracy of between 85% and 115% (LLOQ, 80 to 120%) were accepted in our study.

The relative recoveries of mefloquine and enpiroline were determined by comparing the absolute peak areas of blank plasma samples spiked before and after the extraction at 0.75, 7.5, and 30 μg/ml ($n = 5$ per

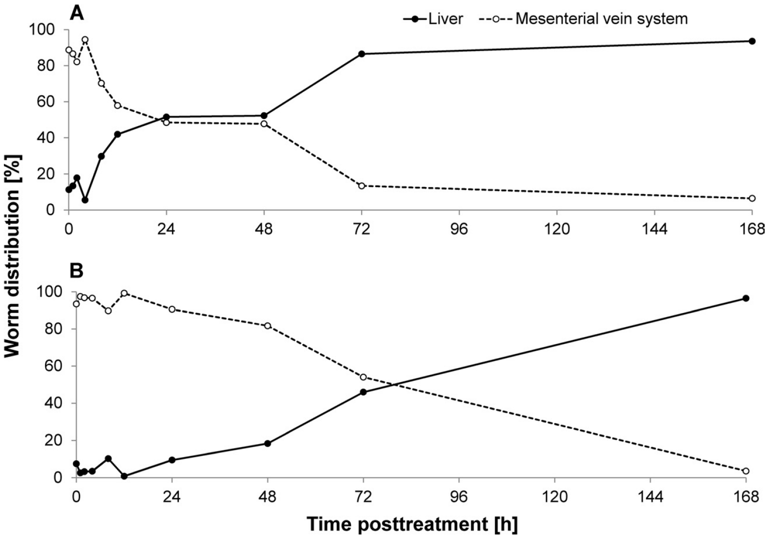

FIG 2 Worm distribution (live and dead) between liver and mesenterial vein system presented over time posttreatment with 200 mg/kg mefloquine (A) or enpiroline (B).

concentration). The matrix effects of mefloquine and enpiroline were assessed as the ratios of the absolute peak areas of blank plasma samples spiked after the extraction to the absolute peak areas of the analytes solved in a mixture of acetonitrile-KH_2PO_4 buffer (1:1, vol/vol).

Autosampler stability and extended bench-top stability studies were included in our method validation. QC samples were used to test stabilities under different conditions. Autosampler stability was evaluated by the analysis of QC samples ($n = 6$ per concentration) over a period of 72 h. Extended bench-top stability was evaluated after samples were kept under bench-top conditions for 8 h. The concentrations of these samples were compared with the concentrations of freshly prepared QC samples. The drug solutions were considered stable with deviations of not more than ±15% and ±20% at the LLOQ.

For the HPLC-UV detection, an aliquot (50 μl) of each sample was injected onto an Inertsil 8-3 analytical column (2.1 mm by 30 mm, 3.5 μm; GL Sciences, Japan). Separation was carried out using an isocratic elution method with a mobile phase consisting of 35% methanol, 25% acetonitrile, and 40% KH_2PO4 buffer (0.05 M) adjusted to pH 3.9 with 0.05% phosphoric acid. The flow rate was set at 1 ml/min and increased to 1.5 ml/min from 11 min onwards. UV signals were detected at 284 nm. The Agilent 1100 series HPLC system (Agilent Technologies, Inc.) consisted of binary pumps, a microvacuum degasser, an analytics autosampler, a column heater (temp, 25°C), and a UV-vis detector.

Pharmacokinetic analysis and statistics. The analytical raw data (area under the concentration-time curve [AUC]) of the plasma samples were processed with ChemStation software (Agilent). Average values, as well as calibration lines and animal data, were calculated using Microsoft Excel 2007. Statsdirect (Statsdirect Ltd., Altrincham, Cheshire, United Kingdom) was used to determine whether worm burden reductions were significant (Kruskal-Wallis test). The mean value from the three animals at each time point was plotted against time to give plasma concentration-time profiles. The PK parameters of mefloquine and enpiroline were determined by noncompartmental analysis using WinNonLin (version 5.2, Pharsight Corporation, USA). The following parameters are presented here: maximal plasma concentration (C_{max}; ng/ml), time to achieve maximal plasma concentration (T_{max}; h), AUC from 0 to 168 h ($AUC_{0\text{-}168}$; ng · ml/min), AUC extrapolated to infinity ($AUC_{0\text{-}\infty}$; ng · ml/min), and terminal elimination half-life ($t_{1/2}$; h). T_{max} and C_{max} are the observed data from the mean concentration time curve. The terminal elimination rate constant is the first-order rate constant associated with the terminal elimination phase. It is estimated via linear regression of time versus log concentration. The extrapolated times and areas after the last data point are extrapolated to infinity. For any of the AUC calculation methods, the AUC rule after the last time point is the log rule.

RESULTS

Studies on the onset of antischistosomal action of enpiroline and mefloquine in mice. We studied the worm distribution from 1 h to 168 h after treatment. Following mefloquine administration, schistosomes started to migrate into the liver (hepatic shift) 8 to 12 h posttreatment. Approximately half of the worms (live and dead) were found within the liver 1 to 2 days posttreatment, and nearly all worms (86.5%) were observed in the liver from day 3 onwards (Fig. 2). A worm burden reduction of 79.2% was calculated at 72 h posttreatment, while a worm burden reduction of 46.9% was determined for the group of mice ($n = 3$) analyzed at 1 week posttreatment.

Worms shifted into the liver slowly following enpiroline treatment, and an equal distribution between liver and mesenterial veins was reached 72 h posttreatment. The majority of worms had migrated to the liver 1 week posttreatment. Enpiroline evolved its full antischistosomal activity 1 week posttreatment, revealing a significant worm burden reduction of 93.1%.

Validation of the HPLC-UV method. The calibration curves of mefloquine and enpiroline were linear from 200 to 40,000 ng/ml mouse plasma ($R^2 > 0.999$). The applied method was found to be selective, since there was no interference between the retention times of analytes and any endogenous substances (signal/noise ratio of >5:1) or any mefloquine metabolites tested (human and animal metabolites). The mean extraction recoveries estimated for mefloquine and enpiroline were 76.6% and 71.3%, respectively, and were consistent over the whole calibration line with a CV of less than 12%. Only minor matrix effects (<20%) were present (Table 1).

The intraday accuracy estimated for both analytes ranged from

TABLE 1 Relative recovery and matrix effect of mefloquine and enpiroline extracted from mouse plasma samples by protein precipitation with methanol

Analyte	Nominal concn (ng/ml)	Relative recovery (%)	Mean ± CV[a] (%)	Matrix effect (%)	Mean ± CV[a] (%)
Mefloquine	750	78.4	76.6 ± 7.9	86.2	83.9 ± 7.1
	7,500	72.9		77.5	
	30,000	78.3		88.1	
Enpiroline	750	66.3	71.3 ± 12	90.7	84.3 ± 11.1
	7,500	69.8		75.2	
	30,000	77.9		87.0	

[a] $n = 5$ samples.

TABLE 3 Pharmacokinetic parameters of mefloquine and enpiroline following oral administration to mice infected or not infected with *S. mansoni*

	Mean value ($n = 3$ mice/time point) for mice treated (200 mg/kg) with:			
	Mefloquine		Enpiroline	
Parameter	*S. mansoni* infected	Noninfected	*S. mansoni* infected	Noninfected
$t_{1/2}$ (h)	182.7	33.6	182.1	72.0
AUC_{0-168} (ng · h/ml)	567,845.5	503,623.5	819,557.5	859,603
$AUC_{0-\infty}$ (ng · h/ml)	1,116,517.8	522,409.1	1,790,287.1	1,107,982.3
C_{max} (ng/ml)	7,238	10,223	5,746	7,641
T_{max} (h)	12	12	24	48

87.8% to 111.4%, with a precision of ≤10%. Interday accuracy was always within a 90 to 110% margin, with a maximal imprecision of 10.5% (Table 2). The LLOQ value of 200 ng/ml plasma was determined with interday accuracies of 97.4% ± 3.8% for mefloquine and 113.5% ± 4.4% for enpiroline.

The results obtained from stability experiments demonstrated that the samples were stable for increased times under bench-top conditions ($t = 8$ h) and autosampler conditions (10°C, $t = 72$ h). Variations of plasma samples following 72 h under autosampler conditions were below 2.9%, and accuracies ranged from 90.2 to 103.5%. Stability was likewise observed for our samples when left at room temperature (23 to 25°C) for 8 h.

Disposition of enpiroline and mefloquine in *S. mansoni*-infected and uninfected mice. The established HPLC-UV method was used to study the pharmacokinetics of mefloquine and enpiroline in *S. mansoni*-infected and noninfected NMRI mice following the administration of a single oral dose of 200 mg/kg mefloquine or enpiroline. The pharmacokinetic parameters are summarized in Table 3.

A 2-fold increase of the $AUC_{0-\infty}$ for mefloquine and enpiroline was observed in *S. mansoni*-infected mice. The C_{max} levels were increased 30 to 40% within the noninfected cohorts (10,223 ng/ml versus 7,238 ng/ml for mefloquine [$R^2 = 0.94$ to 0.98] and 7,641 ng/ml versus 5,746 ng/ml for enpiroline [$R^2 = 0.99$]). The time to achieve maximal concentration (T_{max}) altered only for enpiroline, with T_{max} being reached 24 h later in the noninfected animals than in infected mice (24 versus 48 h). The half-lives ($t_{1/2}$) of mefloquine and enpiroline were elevated 5- and 2.5-fold, respectively (182.7 h versus 33.6 h for mefloquine and 182.1 h versus 72 h for enpiroline), in the infected population. The plasma concentration-time profiles of both drugs in infected and uninfected mice are presented in Fig. 3.

DISCUSSION

Chronic *S. mansoni* infections cause substantial pathological and physiological changes in infected patients, leading to clinical symptoms such as abdominal pain, diarrhea, blood in the stool, and finally, liver cirrhosis and portal hypertension (18).

The liver and gut, which are affected the most by *S. mansoni* infection, play important roles in drug metabolism and disposition (3, 19). Changes in the absorption, distribution, metabolism, and elimination of orally applied drugs are therefore very likely in *S. mansoni*-infected individuals. An altered drug disposition has been shown in several clinical trials in patients with schistosomiasis (6, 7).

In the past few years, drug metabolism and PK studies have increasingly been integrated into early stages of the drug discovery process. However, in the field of schistosomiasis, few studies have been carried out to date elucidating the pharmacokinetic/pharmacodynamic (PK/PD) relationships and studying the influence of the parasitic infection on drug disposition. It was therefore our aim to investigate the PK properties of the two antimalarials mefloquine and enpiroline in *S. mansoni*-infected and uninfected animals.

We successfully adapted an analytical HPLC-UV method, originally used to determine artesunate, its active metabolite dihydroartemisinin, and mefloquine in human plasma (15), to analyze enpiroline and mefloquine in mouse plasma. The accuracy, precision, and sensitivity values documented for our analytical method were similar to those obtained in the original work.

TABLE 2 Intraday and-interday accuracy and precision[a]

		Intraday values for:			Interday values for:		
Analyte (regression model)	Nominal concn (ng/ml)	Mean concn (ng/ml)	RSD (%)	Accuracy (%)	Mean concn (ng/ml)	RSD (%)	Accuracy (%)
Mefloquine (linear, $R^2 = 0.9995$)	750	835.3	1.8	111.4	732.0	9.5	97.6
	7,500	6,779.9	2.9	90.4	7,625.3	8.9	101.7
	30,000	29,129.3	2.0	97.1	31,222.4	6.4	104.1
Enpiroline (linear, $R^2 = 0.9996$)	750	816.9	5.0	108.9	797.2	8.9	106.3
	7,500	6,584.6	5.4	87.8	7,762.6	10.5	103.5
	30,000	28,809.1	2.2	96.0	31,905.2	8.0	106.4

[a] Intraday values are the mean values of 6 samples. The results of one representative experiment are shown. Interday values are the mean values of 3 independent sets of experiments. RSD, relative standard deviation.

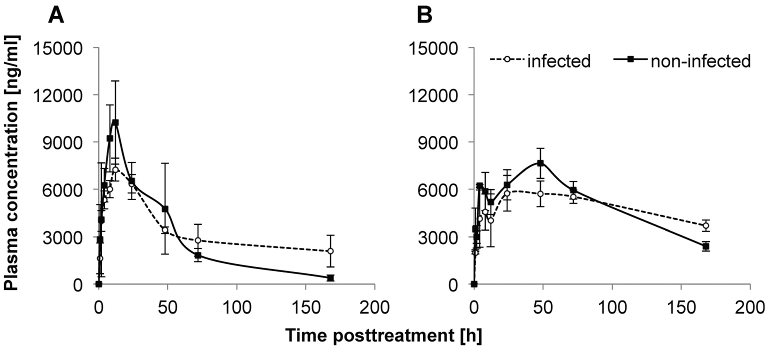

FIG 3 Mean plasma concentration-time profiles of mefloquine (A) and enpiroline (B) after oral administration of drug at 200 mg/kg to 3 mice per time point for either infected or noninfected animals. Error bars show standard deviations.

The speed of onset of action represents a key pharmacodynamic parameter. We determined the hepatic shift (a well-described parameter in antischistosomal drug discovery), which monitors the forced migration of schistosomes to the liver after treatment with active drugs (20). Interestingly, the hepatic shift of all worms started rather late, only 72 h posttreatment for mefloquine and 168 h following drug administration for enpiroline. The hepatic shift of mefloquine observed in our study was comparable to those in two previous studies in *S. mansoni*-infected mice using a single oral dose of 400 mg/kg (21, 22). Mefloquine acts slightly faster in *Schistosoma japonicum*-infected mice (22, 23). The slow onset of action of mefloquine on schistosomes *in vivo* has been reported in other studies (23). In contrast, praziquantel acts much faster; the hepatic shift was already completed 30 min after oral drug administration (unpublished observations), which might be explained by the drug's action on the muscles of schistosomes. In addition, *in vivo* studies showed that extensive structural changes to worms occurred within 15 min after treatment and adult worms died within 24 h following treatment with praziquantel (21, 24).

Interestingly, the majority of worms exposed to mefloquine were still not affected at 7 days (reduction in worm burden, 46.9%) posttreatment in our study. On the other hand, previous studies presented worm burden reductions of 74.1% already at 1 week posttreatment with mefloquine (400 mg/kg), and a worm burden reduction of 72.3% at 2 weeks posttreatment was observed for the dosage of 200 mg/kg (22). These differences in worm burden reductions might be explained by the rather small groups of three mice per time point in our study setup. Additionally, the speed of action of the 200 mg/kg dosage might be slower.

The rather late onsets of action might correlate with the long half-lives determined for mefloquine ($t_{1/2}$, 182.7 h) and enpiroline ($t_{1/2}$, 182.1 h) in infected animals, which are increased as much as 2.5- to 5-fold compared to those in noninfected animals. A previous PK study with mefloquine in uninfected mice revealed a half-life of 17 h after oral treatment with 8 to 10 mg/kg, which is in a range similar to that described from our estimation of 33.6 h (25). However, it is important to emphasize that a rather small sample size was used in the present work and interindividual variations between the animals are high. Furthermore, the estimated AUC is 2-fold higher in infected animals than in noninfected mice for both drugs. The slow clearance of both drugs might be attributed to a decreased activity of the cytochrome P450 enzyme system caused by the *S. mansoni* infection (8). It is known that the AUC and the elimination half-life of mefloquine in humans are significantly increased by inhibition of CYP3A4 with inhibitors (e.g., ketoconazole) (Lariam information leaflet). Surprisingly, both drugs presented rather decreased maximal plasma concentrations (C_{max}) in infected mice. The heavy inflammation of the gut wall that was observed microscopically might result in decreased drug absorption, which could be an explanation for this finding. It is interesting to note that, contrary to our findings, increased C_{max} values were determined for praziquantel in infected rodents (8).

Mefloquine presented plasma concentrations above the IC_{50} (half-maximal inhibitory concentration) value determined *in vitro* (4.7 ± 2.8 μg/ml at 24 h postexposure) for approximately 32 h (12). Interestingly, the plasma levels of enpiroline did not reach the IC_{50} determined at 24 h *in vitro* (7.4 ± 2.7 μg/ml). However, enpiroline is a slow-acting drug *in vitro*, presenting an IC_{50} of 3.1 μg/ml after 72 h of drug exposure (12). The plasma level remained above this concentration for up to 150 h. Since C_{max} levels are reached much earlier (12 h for mefloquine and 24 h for enpiroline) than the onset of action (24 to 72 h for mefloquine and 168 h for enpiroline), one can speculate that the antischistosomal activities of both drugs are AUC rather than C_{max} driven. However, further rigorous PK/PD studies are required to strengthen our hypothesis.

In conclusion, we showed that an *S. mansoni* infection triggers considerable changes in the drug disposition of mefloquine and enpiroline. Pharmacokinetic studies with antischistosomal lead candidates in infected animals should be integrated early in the drug discovery process. In addition, PK studies should be conducted in *S. mansoni*- and *S. haematobium*-infected children, the key target group of preventive chemotherapy, since children depend greatly on safe, efficacious, and controlled dosage regimens. Our findings might be of public health relevance, since mefloquine is widely used in areas where schistosome infections are common. Overdosing might occur and lead to toxic adverse events, which might be of relevance in light of the reported neurotoxicity of mefloquine (26). Furthermore, influences on the pharmacokinetic profiles of antimalarials could lead to prolonged plasma levels beneath the minimal inhibition concentration and, hence, result in suboptimal dosing and selection for drug-resistant parasites. Therefore, in-depth studies should also be conducted with antimalarials in patients coinfected with *S. mansoni* and *Plasmodium falciparum*.

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Chapter 5

Preclinical studies of synthetic peroxides

5.1 In vivo Activity of Aryl Ozonides against *Schistosoma* species

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In Vivo Activity of Aryl Ozonides against *Schistosoma* Species

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We evaluated the *in vivo* antischistosomal activities of 11 structurally diverse synthetic peroxides. Of all compounds tested, ozonide (1,2,4-trioxolane) OZ418 had the highest activity against adult *Schistosoma mansoni*, with total and female worm burden reductions of 80 and 90% ($P < 0.05$), respectively. Furthermore, treatment of *S. haematobium*-infected mice with OZ418 reduced the total worm burden by 86%. In conclusion, OZ418 is a promising antischistosomal lead compound.

Schistosomiasis, caused by blood flukes of the genus *Schistosoma*, is an important cause of morbidity and mortality, mainly among the rural poor in sub-Saharan Africa (5, 16). The need for new antischistosomal drugs is quite evident, given that praziquantel is the only drug available and is in widespread use in population-based morbidity control programs (16).

Various classes of synthetic peroxides, including trioxaquines (1,2,4-trioxanes) (1, 12), the tetraoxaspirononadecane N-89 (15), and ozonides (1,2,4-trioxolanes) (19), have been studied for their antischistosomal properties. Our previous data demonstrate that, like the artemisinins, ozonides possess promising antischistosomal properties (10, 19). We investigated three representative ozonides, namely, carboxylic acid OZ78, amine OZ209, and phenol OZ288 (Fig. 1). Of these, 8′-aryl ozonide OZ288 had the highest antischistosomal activity; this ozonide reduced the total worm burden by 95% (200 mg/kg body weight) in mice harboring juvenile *Schistosoma mansoni* and by 52% (400 mg/kg) in mice harboring adult worms (19). Although 8′-alkyl ozonides OZ78 and OZ209 had activities greater than 80% against juvenile worms, lower activities (0 and 17%, respectively) were observed for these two ozonides against adult worms. We suggest that the increased iron(II) stabilities of 8′-aryl ozonides compared to those of 8′-alkyl ozonides (2) might contribute to the superior antischistosomal efficacy of OZ288.

The aim of the present study was to evaluate in a first step the *in vivo* antischistosomal activities of nine structurally diverse OZ288 analogs (Fig. 2), including antimalarial drug candidate OZ439, currently in phase II clinical testing (2). Since schistosomiasis and malaria are coendemic in many parts of the world, it is important to assess the potential auxiliary effects of antimalarial drug candidates such as OZ439 on *Schistosoma* species.

Our animal studies were carried out following national and cantonal regulations on animal welfare (permission no. 2070). All compounds were synthesized as described in previous publications and patents (3, 4, 17, 18). Compounds were freshly prepared in homogenous aqueous suspensions in 7% Tween 80 and 3% ethanol.

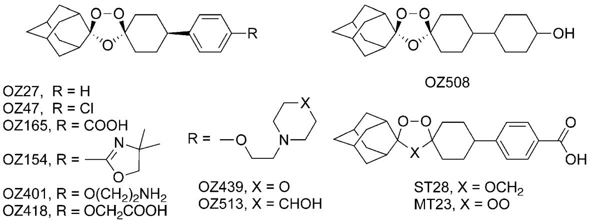

FIG 2 Structures of OZ288 analogs, 1,2,4-trioxane ST28, and 1,2,4,5-tetraoxane MT23.

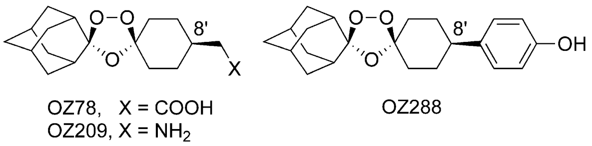

FIG 1 Structures of ozonides OZ78, OZ209, and OZ288.

Female NMRI mice ($n = 147$; age, 4 weeks; weight, ~20 g) were purchased from Charles River (Sulzfeld, Germany) and Harlan Laboratories (Blackthorn, United Kingdom). Mice were maintained in groups of 10 in Makrolon cages under environmentally controlled conditions (temperature, ~25°C; humidity, ~50%; 12-h light and 12-h dark cycle) with free access to water and food. Mice were acclimatized for 1 week prior to infections.

Each mouse was infected subcutaneously with ~80 *S. mansoni* cercariae. Twenty-one days (pre-patent infection) or 49 days (patent infection) after the experimental infection, groups of 3 to 5 mice were treated orally with the test drugs at 400 mg/kg (adult infection) or 200 mg/kg (juvenile infection). In order to assess the effect of OZ418 against *S. haematobium*, 16 mice were similarly infected with ~300 *S. haematobium* cercariae per mouse. Ninety days postinfection (patent infection), mice ($n = 4$) were treated orally with 400 mg/kg OZ418. Infected untreated mice served as controls in both experiments. By use of CO_2, *S. mansoni*- and *S. haematobium*-infected mice were killed 21 days posttreatment; they were dissected, and schistosomes were sexed and counted. For statistical analysis, the Kruskal-Wallis (KW) test was used

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TABLE 1 Effect of selected ozonides administered at single oral doses of 400 mg/kg against adult *S. mansoni* worms harbored in NMRI mice

Compound	Total worm burden reduction (%)	Female worm burden reduction (%)
Praziquantel[a]	96	98
OZ288[b]	52	65
OZ27	20	ND[c]
OZ47	12	8
OZ154	0	8
OZ165	74	78
OZ401	0	5
OZ418	80	90
OZ439	35	43
OZ508	0	0
OZ513	24	23

[a] Total worm burden reduction reported in reference 7.
[b] Based on the study of Xiao et al. in 2007 (19).
[c] ND, not determined.

(Statsdirect statistical software, version 2.4.5; Cheshire, United Kingdom).

The antischistosomal activities of the nine OZ288 analogs against adult *S. mansoni* are summarized in Table 1. For comparison, data obtained with the drug of choice, praziquantel, are presented (7).

In contrast to OZ288, the ozonides with neutral and weak base functional groups either were completely inactive or showed very weak activities (5 to 43%). This, combined with the lack of activity of OZ508, demonstrate that the 8′-aryl substructure of OZ288 is a necessary but insufficient requirement for antischistosomal activity. Total and female worm burden reductions of 35 and 43%, respectively, were documented for the antimalarial drug candidate OZ439. The only ozonides more effective than OZ288 were carboxylic acids OZ165 and OZ418. We also note that in contrast to the neutral and weak base ozonides (Fig. 2) with 50% inhibitory concentrations (IC_{50}s) less than 5 ng/ml against cultured *Plasmodium falciparum* (2, 4, 17), these two acidic ozonides are much less potent against *P. falciparum*, with respective IC_{50}s of 37 and 97 ng/ml (4, 17).

Since the highest activity was observed with OZ165 and OZ418, these ozonides, along with the 1,2,4-trioxane (ST28) and 1,2,4,5-tetraoxane (MT23) analogs of OZ165 (Fig. 2), were assessed in more detail (Table 2). Each of the four compounds achieved high worm burden reductions (82 to 100%) in mice infected with juvenile *S. mansoni*, consistent with previous observations that synthetic peroxides have high activities against the juvenile stage of the parasite (1, 19). However, the four compounds had significant differences in efficacy against adult *S. mansoni*. For example, low total and female worm burden reductions of 33 and 40% were observed with ST28 ($P > 0.05$). Treatment of mice with MT23 resulted in moderate total and female worm burden reductions of 62 ($P = 0.037$) and 57% ($P > 0.05$), respectively. A minor shift of worms from the mesenteric veins to the liver was observed with these two compounds. Treatment with OZ165 achieved significant total and female worm burden reductions of 74 and 78%. Thus, for this set of peroxide heterocycles, OZ165 was more effective than either ST28 or MT23. In contrast, we have recently shown (18) that ST28 is more effective than either OZ165 or MT23 against *Fasciola hepatica*, another pathogenic liver fluke. Of all of the compounds tested, OZ418 had the highest antischistosomal efficacy, with total and female worm burden reductions of 80 and 90%, respectively (both P values = 0.01). Note that worm counts (mean worm counts of 13 to 28 worms) in the present work were slightly lower than in previous studies (mean worm counts of 39 to 41 worms) (19). In this respect, clinical studies recently demonstrated that significantly higher cure rates were

TABLE 2 Effect of 1,2,4-trioxolanes (ozonides) OZ165 and OZ418, 1,2,4-trioxane ST28, and 1,2,4,5-tetraoxane MT23 against juvenile and adult *S. mansoni* worms harbored in NMRI mice

Infection (dose)	Compound tested	No. of mice investigated	No. of mice cured	Mean no. of worms (SD)[e] Liver	Mean no. of worms (SD)[e] Mesenteric veins	Mean no. of worms (SD)[e] Males	Mean no. of worms (SD)[e] Females	Total worm burden reduction %	Total worm burden reduction P value	Female worm burden reduction %	Female worm burden reduction P value
None	Control 1	10	0	1.9 (1.7)	26.0 (13.1)	15.4 (8.1)	12.5 (7.0)				
	Control 2	9	0	0.9 (1.1)	16.6 (4.9)	8.5 (3.3)	8.9 (3.4)				
	Control 3	18	0	1.8 (2.3)	21.6 (13.3)	12.8 (7.4)	10.5 (6.6)				
	Control 4	18	0	1.2 (2.3)	17.4 (13.7)	6.9 (5.0)	6.1 (5.0)				
Adult infection (400 mg/kg)	OZ165[c]	6	0	1.2 (1.2)	4.8 (6.6)	3.6 (3.7)	2.3 (3.8)	74.1	0.0029	78.1	0.003
	ST28[a]	4	0	3.3 (2.5)	15.0 (6.7)	11.3 (3.2)	7.5 (3.8)	32.8	0.228	40.0	0.117
	MT23[a]	5	1	2.6 (2.6)	8.0 (10.3)	5.2 (5.0)	5.4 (5.8)	62.1	0.037	56.8	0.097
	OZ418[d]	5	0	0.5 (0.6)	2.0 (0.8)	2.0 (0.7)	0.6 (0.6)	80.0	0.01	90.2	0.01
Juvenile infection (200 mg/kg)	OZ165[b]	4	0	0.8 (0.5)	2.0 (2.3)	1.75 (1.0)	1.0 (1.2)	84.2	0.005	88.8	0.005
	ST28[a]	4	0	1.0 (1.4)	2.5 (1.0)	1.25 (0.5)	2.3 (1.9)	87.5	0.005	81.6	0.006
	MT23[a]	4	0	1.5 (1.3)	0.5 (1.0)	0.5 (0.6)	1.5 (1.3)	92.8	0.005	88.0	0.005
	OZ418[d]	4	4	0	0	0	0	100	0.005	100	0.005

[a] Data for this compound are relative to control 1.
[b] Data for this compound are relative to control 2.
[c] Data for this compound are relative to control 3.
[d] Data for this compound are relative to control 4.
[e] SD, standard deviation.

TABLE 3 Effects of ozonide OZ418 and praziquantel against adult *S. haematobium* worms harbored in NMRI mice

			Mean no. of worms (SD)			Total worm burden reduction	
Compound tested	No. of mice investigated	No. of mice cured	Liver	Mesenteric veins	Total	%	*P* value
Control	12	0	2.3 (4)	3 (4)	5.3 (5)		
OZ418 (400 mg/kg)	4	2	0 (0)	0.8 (1)	0.8 (1)	85.9	0.01
Control	14	0	2.1 (3.7)	2.7 (2.6)	4.9 (3.9)		
Praziquantel (200 mg/kg)	4	3	0 (0)	0.25 (0.5)	0.25 (0.5)	94.9	0.003

observed with the artemisinins in patients with lower infection intensities of *Fasciola* spp. and *Schistosoma* spp. (8, 11).

Given the superior efficacy of OZ418, we tested this ozonide against *S. haematobium*, another major human-pathogenic schistosome species (14). Treatment of *S. haematobium*-infected mice with OZ418 resulted in significant ($P = 0.01$) total worm burden reduction of 86% (Table 3), demonstrating that OZ418 has high efficacy against two schistosome species.

In conclusion, OZ418 is a promising lead compound effective against several developmental stages of *S. mansoni*. The activity of OZ418 against adult *S. mansoni* compares favorably with that of praziquantel (50% effective dose [ED_{50}] of 172 mg/kg; worm burden reduction of 96% at 400 mg/kg) (Table 1) (7). In contrast to praziquantel, which lacks activity against juvenile worms (6), OZ418 also has high activity against the juvenile stage of *S. mansoni*. Importantly, this compound is also quite effective against *S. haematobium*, the causative agent of urinary schistosomiasis. Accordingly, OZ418 possesses many properties of an ideal antischistosomal compound (9, 13). Given the threat of praziquantel resistance and lack of orally active antischistosomal candidates in the drug development pipeline that show high activities against adult and juvenile schistosomes and are active against multiple schistosome species, we continue to investigate OZ418 and its analogs as potential anthelmintic drug development candidates.

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5.2 In vitro and in vivo activity of 3-alkoxy-1,2-dioxolanes against *Schistosoma mansoni*

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In vitro and *in vivo* activity of 3-alkoxy-1,2-dioxolanes against *Schistosoma mansoni*

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Objectives: Compounds characterized by a peroxidic skeleton are an interesting starting point for antischistosomal drug discovery. Previously a series of 3-alkoxy-1,2-dioxolanes, which are chemically stable cyclic peroxides, demonstrated significant *in vitro* activity against *Plasmodium falciparum*. We aimed to evaluate the potential of these compounds against *Schistosoma mansoni* and elucidate the roles of iron and peroxidic groups in activity.

Methods: Drugs were tested against juvenile and adult stages of *S. mansoni in vitro* and *in vivo*. Selected structures were assessed *in vitro* against schistosomes in the presence of additional iron sources. In addition, drugs were tested *in vitro* and *in vivo* against *Echinostoma caproni*, a non-blood-feeding intestinal fluke. Finally, the activity of non-peroxidic analogues was evaluated.

Results: Three dioxolanes displayed IC_{50}s $\leq$20.1 μM against adult schistosomes and values as low as 4.2 μM against newly transformed schistosomula. Nonetheless, only moderate, non-significant worm burden reductions were observed after treatment of mice harbouring adult infections. Drugs lacked activity against juvenile schistosomes *in vivo*. Two selected dioxolanes showed *in vitro* activity against *E. caproni* down to concentrations of 5 mg/L, but none of the compounds revealed *in vivo* activity. All tested non-peroxidic analogues lacked activity *in vitro* against both parasites.

Conclusions: Selected dioxolanes presented interesting *in vitro* activity, but low *in vivo* activities have to be overcome to identify a lead candidate. Although the inactivity of non-peroxidic analogues underlines the necessity of a peroxide functional group, incubation of adult schistosomes with additional iron sources did not alter activity, supporting an iron-independent mode of activation.

Keywords: chemotherapy, peroxides, non-peroxidic analogues, schistosomiasis

Introduction

Schistosomiasis is a neglected tropical disease caused by trematode flatworms of the genus *Schistosoma*. Five species of schistosomes infect humans, with *Schistosoma haematobium*, *Schistosoma japonicum* and *Schistosoma mansoni* being responsible for the main burden of schistosomiasis.[1] Approximately 200 million people are affected by schistosomiasis, mainly in sub-Saharan Africa.[2] Praziquantel is currently the gold standard for the treatment of schistosome infections. Because of the threat of drug resistance and the limitations in the activity profile of praziquantel (the drug lacks activity against the juvenile schistosome stages), identification of potential drug candidates has a high priority.

The antischistosomal potential of the antimalarials artemisinin and its semisynthetic derivatives artesunate and artemether has been studied thoroughly in the past three decades, in *in vitro* and *in vivo* studies, and in clinical trials.[3–5] Given the promising activity profile of the artemisinins, with particularly high activities observed against juvenile schistosomes, different groups of fully synthetic peroxides have been studied *in vivo* and *in vitro* in recent years.[6,7] For example, the synthetic trioxolane OZ418 was recently introduced as a promising drug candidate showing high worm burden reductions (WBRs) of 80% and 86% against *S. mansoni* and *S. haematobium*, respectively.[8]

The high antimalarial activity of 1,2,4-trioxolanes triggered investigations with 1,2-dioxolanes, which are structurally analogous five-membered ring peroxides offering enhanced chemical stability. However, the dioxolanes proved much less active than the corresponding 1,2,4-trioxolanes against *Plasmodium falciparum in vitro* and *Plasmodium berghei in vivo*.[9] This reduction in activity has been attributed to a decreased tendency for scission of the alkoxy radicals derived from Fe(II) activation of the 1,2-dioxolane peroxide;[10] β-scission to generate carbon radicals is considered important for the activity of peroxide antimalarials.[11] 3-Alkoxy-1,2-dioxolanes, which undergo activation by Fe(II) to generate oxygen-substituted alkoxy radicals closely related to the intermediates derived from trioxolanes, generate products indicative of efficient β-scission and have been shown to have promising antimalarial efficacy.[10]

To our knowledge the antischistosomal activity of the 3-alkoxy-1,2-dioxolanes has not been studied to date. In the present work 18 selected 3-alkoxy-1,2-dioxolanes were tested on *S. mansoni in vitro* and active candidates were followed up *in vivo*. To investigate whether the peroxide pharmacophore is an essential requirement for antischistosomal activity, non-peroxidic analogues of active compounds were synthesized and their antischistosomal potential was determined. In addition, the relationship between the parasite's haemoglobin consumption and the antischistosomal activity of compounds was studied *in vitro* by testing active compounds on the non-blood-feeding foodborne trematode *Echinostoma caproni* and evaluating the *in vitro* activity of active compounds under different incubation conditions, in media containing haemin, haemoglobin or red blood cells.

Materials and methods

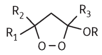
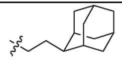
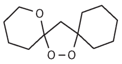
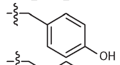
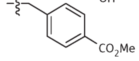
Drugs

The 18 3-alkoxy-1,2-dioxolane substrates illustrated in Table 1 were prepared based upon methods described by Schiaffo *et al.*[10] Two non-peroxidic analogues were prepared as illustrated in Figure S1 (available as Supplementary data at *JAC* Online).

Animals and parasites

Animal studies were conducted following Swiss regulations on animal welfare at the Swiss Tropical and Public Health Institute (Basel, Switzerland, permission no. 2070). Three-week-old (weight ~14 g) female NMRI mice were purchased from Charles River (Sulzfeld, Germany) or Harlan Laboratories (Horst, The Netherlands). Before starting experiments, animals were allowed to adapt for 1 week under controlled conditions (temperature ~22°C; humidity ~50%; 12 h light and 12 h dark cycle; free access to rodent diet and water). Infection of mice with *S. mansoni* (Liberian strain) was performed subcutaneously by injection

Table 1. Chemical structures of investigated 3-alkoxy-1,2-dioxolanes and determined IC_{50} values (mean of three experiments) against newly transformed schistosomula (NTS) and adult *S. mansoni* 72 h post-compound exposure; *r* represents goodness of fit (conformity with $r \geq 0.85$)

					IC_{50} (μM) for *S. mansoni*			
Compound	R_1	R_2	R_3	R	NTS	*r*	adult	*r*
1	Me	Me	Me		31.1	0.9	11.9	1.0
2	Me	Bu	Me	CH_2CH_2Ph	7.5	1.0	12.4	1.0
3	-$(CH_2)_5$-		CH_2CH_2Ph	CH_2CH_2Ph	4.2	0.9	20.1	0.8
4	Me	Me	CH_2Ph	Me	101.0	0.7	49.2	1.0
5					17.0	0.7	76.4	1.0
6	Me	Me	Me	5,6-epoxyhexyl	35.9	0.9	101.5	1.0
7	Me	Me	Me	CH_2CH_2CN	89.2	1.0	137.0	1.0
8	-$(CH_2)_5$-		Me	$CH(OH)CH_2OH$	90.9	0.9	138.4	1.0
9	Me	Me	Me	$(CH_2)_9CO_2Me$	89.7	0.9	169.3	0.9
10	Me	Me	Me	$CH_2CH_2CO_2Me$	76.0	1.0	206.2	1.0
11	Me	Me	Me	CH_2CH_2OMe	69.2	0.8	207.4	0.9
12	Me	Me	Me	$CH_2CH_2\ CH_2OH$	42.6	0.8	235.8	0.9
13	Me	Me	Me	$(CH_2)_4CO_2Me$	59.8	1.0	283.3	0.6
14	Me	Me	Me	$CH(OH)CH_2OH$	102.5	0.9	>436	
15	Me	Me	Me	CH_2CH_2Ph	2.7	0.9	58.4	0.9
16	-$(CH_2)_5$-		Me	CH_2CH_2Ph	2.8	0.9	49.4	0.9
17	-$(CH_2)_5$-		Me		4.1	0.9	71.8	1.0
18	Me	Me	Me		4.8	0.9	109.3	0.9
Praziquantel					2.2	0.9	0.08[a]	
Artesunate					5.0	0.9	41.2	0.8

[a] As described by Keiser *et al.*[26]

of 80–100 cercariae. Cercariae were harvested from infected intermediate host snails (*Biomphalaria glabrata*) by exposure to light for 3 h, following the standard procedures of our laboratory. For *in vitro* and *in vivo* studies with *E. caproni*, mice were infected intragastrically with 30 metacercariae harvested from infected *B. glabrata* snails.

In vitro screening

Preparation of NTS and adult schistosomes

NTS were obtained by mechanical transformation of *S. mansoni* cercariae.[12,13] Cercariae were collected as described above. The schistosomula suspension was adjusted to a concentration of 100 NTS per 50 μL with Medium 199 (Invitrogen, Carlsbad, CA, USA) supplemented with 5% heat-inactivated fetal calf serum (iFCS), 100 U/mL penicillin and 100 mg/L streptomycin (Invitrogen). To ensure completed conversion from cercariae to NTS, suspensions were incubated at 37°C in an atmosphere of 5% CO_2 in ambient air for a minimum of 12–24 h until use.[14]

Adult flukes were harvested from hepatic portal veins and mesenteric veins of infected NMRI mice (7–8 weeks post-infection) following standard procedures.[6] Schistosomes were placed in RPMI 1640 culture medium supplemented with 5% iFCS, 100 U/mL penicillin and 100 mg/L streptomycin at 37°C in an atmosphere of 5% CO_2 until use.

Preparation of adult E. caproni

Infected mice were killed 2 weeks post-infection. Trematodes were harvested from the excised small intestine and placed in RPMI medium supplemented with 100 U/mL penicillin, 100 mg/L streptomycin and 1% α-D-glucose (Sigma Aldrich, St Louis, MO, USA). Flukes were maintained at 37°C in an atmosphere of 5% CO_2 in ambient air until use.

Drug susceptibility assays with NTS

Drug dilution series with concentrations ranging from 0.37 to 90 mg/L (0.37, 1.1, 3.3, 10, 30, 90 mg/L) were prepared in 96-well flat-bottom plates (BD Falcon, USA) using supplemented medium (with iFCS and antibiotics). Prepared NTS suspension containing 100 NTS per 50 μL was added to each well to yield a total volume of 250 μL per well. The highest DMSO concentration (1.1%) used, diluted in Medium 199, served as control. Plates were incubated at 37°C in an atmosphere of 5% CO_2. NTS were evaluated by microscopic readout (Carl Zeiss, Germany, magnification ×80) with regard to death, changes in motility, viability and morphological alterations 24, 48 and 72 h post-drug exposure. As described previously, drug effects were evaluated using a viability scale.[12,13] Parasite fitness, morphology and motility were classified with scores ranging from 3 (normal activity, no morphological changes) to 0 (all worms dead). Duplicate examinations were performed for each concentration and experiments were repeated at least three times. IC_{50} values of the investigated drugs based on motility scale values obtained at the 72 h timepoint were determined using CompuSyn software (Version 3.0.1, 2007; ComboSyn, Inc.).

Drug susceptibility assay with adult schistosomes

In vitro screening on adult flukes was performed in 24-well flat-bottom plates (BD Falcon, USA). Supplemented RPMI 1640 medium (with iFCS and antibiotics) and drug stock solutions (10 mg/mL) were used to obtain final test concentrations of 1–90 mg/mL (1.1, 3.3, 10, 30, 90 mg/L) in wells with a final volume of 1.4 mL. Finally, three schistosomes of both sexes were added to each well. The highest concentration of DMSO (0.3%) in medium served as control. Twenty-four, 48 and 72 h post-drug exposure, phenotypes were monitored using the motility scale described by Ramirez *et al.*[15] and an inverse microscope (Carl Zeiss, Germany, magnification ×80). Experiments were performed three times. IC_{50} values were calculated with CompuSyn software as described above for NTS (Version 3.0.1, 2007; ComboSyn, Inc.).

Drug susceptibility assay of adult S. mansoni in the presence of haemin, haemoglobin or red blood cells

Lead candidates were incubated as described above, but using different RPMI culture medium conditions. A haemin solution (1.5 mM) was prepared as follows: 50 mg haemin chloride (Fluka Analytical, The Netherlands) was dissolved in 10 mL of 0.1 M NaOH, 0.5 mL of 1 M HCl and 39.5 mL of PBS (pH=7.4). The haemoglobin solution (0.23 mM) was prepared using 750 mg haemoglobin from bovine blood (Sigma Aldrich, USA) dissolved in the same amounts of NaOH, HCl and PBS as used above. Finally, supplemented RPMI media were prepared by adding 8% haemin solution, 10% haemoglobin solution or 2% red blood cells from red blood cell concentrate (blood group A Rhesus positive) to final concentrations in well plates of 120 μM for haemin, 23 μM for haemoglobin or 2% for red blood cells. At timepoints 24, 48 and 72 h post-exposure, phenotypes were monitored using the motility scale as described above using an inverse microscope (Carl Zeiss, Germany, magnification ×80) and data were compared between various incubation conditions.[15]

Isothermal microcalorimetry (IMC) drug assay with adult S. mansoni

Two non-peroxidic analogues (compounds 19, 20) and one selected dioxolane (compound 16) were further characterized using IMC as described by Manneck *et al.*[16] Briefly, heat production and motility (derived from noise amplitudes) of schistosomes were measured using a 48-channel microcalorimeter (model TAM 48; TA Instruments, New Castle, DE, USA) over a period of 5 days. Samples were prepared in glass ampoules with 2900 μL of medium (supplemented RPMI 1640) containing four adult worms. Pre-warmed (37°C) ampoules were placed in channels and equilibration was performed for 12 h until a stable signal was observed. Drug suspensions (concentration of 900 mg/L) in supplemented medium (volume 100 μL) were injected, using 1 mL syringes (BD Plastipak, Becton Dickinson S.A., Madrid, Spain), to reach the final concentration of 30 mg/L per ampoule. Ampoules with dead worms served as the negative control and ampoules with worms treated with the highest concentration of DMSO (0.3%) served as the positive control. Heat flow was recorded as 1 data point per 1 min over at least 120 h. Tests at each concentration were performed three times.

Drug susceptibility assay with adult E. caproni

Assays were prepared in 24-well flat-bottom plates (Costar). Drug dilutions were prepared with drug stock solutions and supplemented RPMI medium (with antibiotics and glucose) to obtain final drug concentrations of 5–100 mg/L (5, 10, 50, 100 mg/L) in a total volume of 2 mL per well. Six to nine trematodes were used (one or two worms per well) for each experimental group. The highest concentration of DMSO (1%) served as control. Plates were incubated at 37°C in an atmosphere of 5% CO_2. Twenty-four, 48 and 72 h post-drug exposure, phenotypes and mortality of worms were monitored as described elsewhere.[12]

In vivo screening

Studies with S. mansoni

Groups of four infected NMRI mice characterized by a patent schistosome infection (49 days post-infection) or a juvenile *Schistosoma* infection

(21 days post-infection) were treated orally with the test drug using single oral doses of 400 mg compound per kg body weight. Seven to nine untreated mice served as controls. Animals harbouring an adult *Schistosoma* infection were killed by the CO_2 method 14 days post-treatment, and mice treated at the juvenile infection stage were sacrificed 4 weeks post-treatment. Mice were then dissected and worms sexed and counted.[6] Worm burdens of treated mice were compared with those of untreated animals and reductions of worm burden calculated.

Studies with E. caproni

Four NMRI mice were treated intragastrically with 400 mg/kg of test compounds 2 weeks post-infection with *E. caproni*. Four mice were left untreated and served as controls. One week post-treatment, mice were euthanized with CO_2. At necropsy, the intestines were removed from the pylorus to the ileocaecal valve, placed in a Petri dish and opened longitudinally. All *E. caproni* worms were removed and counted.

Statistics

Parasite motility of treated and untreated NTS and adult trematodes was calculated as mean ($\pm$SD) using Microsoft Excel software. Motility data obtained from experiments with various media containing iron sources were compared using the Mann–Whitney test (considered significant at $P \leq 0.05$). IC_{50} values were determined using the CompuSyn software (Version 3.0.1, 2007; ComboSyn, Inc.). For the comparison of IC_{50} values we used the Kruskal–Wallis test (considered significant at $P \leq 0.05$). The Kruskal–Wallis test was also used for *in vivo* studies, comparing the medians of the responses between the treatment and control groups. A difference in median was considered to be significant at a significance level of 5% (StatsDirect statistical software, version 2.7.2.; StatsDirect Ltd, UK). Noise amplitudes and heat flows observed in calorimetric *in vitro* assays with adult *S. mansoni* were analysed using R software and Microsoft Excel. As described by Manneck *et al.*,[17] noise amplitude values follow an exponential decay. Endpoints of worm motility were determined by the intersection of the sample amplitude curve with the background signal noise of dead worms.

Results

In vitro screening against S. mansoni

In a first step, 14 3-alkoxy-1,2-dioxolanes were studied against adult *S. mansoni in vitro*. Three of the compounds (1, 2 and 3) demonstrated good antischistosomal activity with IC_{50}s of 11.9, 12.4 and 20.1 μM, respectively, against adult flukes. The remaining 11 substances revealed only minor activity (IC_{50}s between 49.2 and 283.3 μM) or lacked activity (compound 14). Good activities were observed against the juvenile schistosome stage (NTS) for compounds 2 (IC_{50} 7.5 μM) and 3 (IC_{50} 4.2 μM). Moderate activity was detected for compounds 1 (IC_{50} 31.1 μM), 5 (IC_{50} 17.0 μM) and 6 (IC_{50} 35.9 μM) against NTS. Finally, only minor activity was recorded for the remaining nine compounds against NTS (IC_{50} 42.6–102.5 μM). *In vitro* findings are summarized in Table 1. For comparison, activities of standard antischistosomal drugs, praziquantel and artesunate, are also shown in Table 1.

Influence of haemin, haemoglobin or red blood cells on in vitro antischistosomal activity

Incubation of adult schistosomes with various concentrations (1.1, 3.3, 10, 30 mg/L) of the three active alkoxydioxolanes (1–3) in supplemented media containing haemin, haemoglobin or red blood cells showed no significant differences in activity. The motilities recorded 72 h post-exposure of *S. mansoni* with compound 1 at different concentrations did not vary among the different media tested (Figure 1). Slightly higher, though not significant ($P > 0.05$) activities were detected for compounds 2 and 3 when incubated in the presence of haemin or haemoglobin. In the presence of these media all worms had died 72 h post-exposure to the tested compounds at a concentration of 10 mg/L and 30 mg/L.

In vivo activity of selected alkoxydioxolanes against S. mansoni

The three lead structures 1–3 identified by prior *in vitro* screening, were tested in a juvenile as well as an adult *S. mansoni* infection mouse model (Table 2). All studied alkoxydioxolanes lacked *in vivo* activity against juvenile *S. mansoni* (Table 2; WBRs 0%–4%). However, moderate, non-significant activities were observed with compounds 1 and 2 against adult *S. mansoni* with WBRs of 42.5% and 37.0%, respectively. Compound 3 showed only low activity against adult *S. mansoni in vivo* with a WBR of 15.1%. Dead worms were detected in mouse livers after treatment of adult infections with compounds 1, 2 and 3. The presence of a patent schistosoma infection in treated animals was confirmed by observing granulous tissue and *Schistosoma* eggs within all livers.

In vitro and in vivo activity of modified lead dioxolanes against S. mansoni

Given the low *in vivo* activity of the three test drugs, we were motivated to investigate four additional chemically related alkoxydioxolanes *in vitro* against NTS and adult schistosomes, followed by *in vivo* studies on a patent *S. mansoni* infection. All four compounds (15–18) showed very high activities against NTS, represented by low IC_{50} values (2.7–4.8 μM) (summarized in Table 1). However, the four drugs revealed only low activities against adult schistosomes *in vitro*, with IC_{50}s between 49.4 and 109.3 μM. Moderate, but non-significant, *in vivo* WBRs of 32.0% and 21.3% were achieved with compounds 15 and 17, respectively. Low activities with WBRs of 12.0% and 16.4% were observed with compounds 16 and 18, respectively (summarized in Table 3).

In vitro and in vivo effect against E. caproni

Lead structures 1–3 as well as the four related structures (15–18) were tested *in vitro* on freshly harvested *E. caproni*. Data are summarized in Table 4. Six of seven compounds (1, 3, 15–18) showed 100% worm mortality 24 h post-drug exposure at a concentration of 50 mg/L. Two of the compounds (16 and 17) killed all worms 24 h post-incubation at a 5-fold lower concentration of 10 mg/L. Incubation of adult *E. caproni* with three spirocyclohexyl compounds (3, 16 and 17) at 5 mg/L for 72 h resulted in death of all worms. Compounds 15–17 were followed

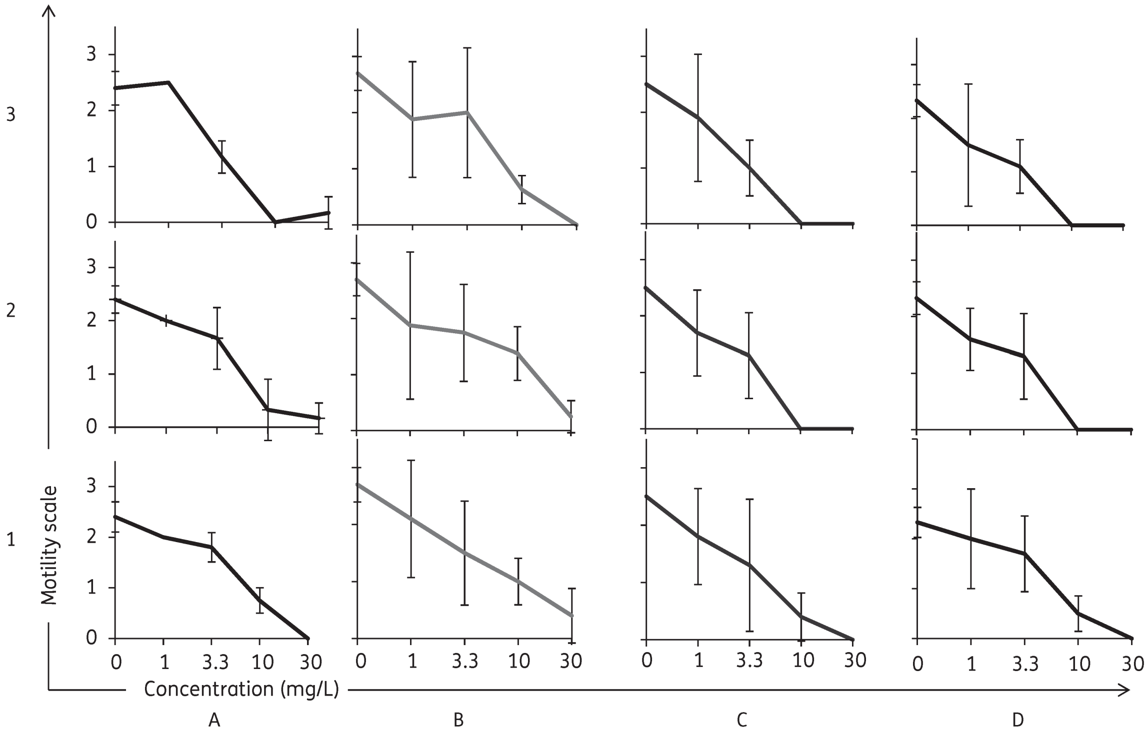


Figure 1. Motility of adult schistosomes 72 h post-treatment with various concentrations (1.1, 3.3, 10 and 30 mg/L) of compounds 1, 2 or 3 in four different incubation settings. (a) Standard incubation with supplemented RPMI. (b) Addition of 2% red blood cells from red blood cell concentrate. (c) Addition of 120 μM haemin. (d) Addition of 23 μM haemoglobin.

Table 2. *In vivo* activity following a single oral dose of 400 mg/kg of selected 3-alkoxy-1,2-dioxolanes in mice harbouring juvenile or adult *S. mansoni*

Group	No. of mice investigated	No. of mice that died	No. of mice cured	TWR (%)	*P* value	FWBR (%)	*P* value
Control	8	0	0	–	–	–	–
Juvenile infection							
1	4	0	0	0	0.73	0	0.86
2	4	0	0	4.1	0.67	5.9	0.38
3	4	0	0	0	0.87	0	0.44
Adult infection							
1	4	0	0	42.5	0.27	47.1	0.23
2	4	0	0	37.0	0.35	41.2	0.35
3	4	0	0	15.1	0.86	17.7	0.73

TWR, total worm burden reduction; FWBR, female worm burden reduction.

up *in vivo*. Treatment of *E. caproni*-infected mice with a single oral dose of 400 mg/kg of compound 15 resulted in a low WBR of 17.4%. Compounds 16 and 17 lacked *in vivo* activity (Table 5).

Table 3. *In vivo* activity following a single oral dose of 400 mg/kg of selected 3-alkoxy-1,2-dioxolanes in mice harbouring adult *S. mansoni*

Group	No. of mice investigated	No. of mice that died	No. of mice cured	TWR (%)	*P* value	FWBR (%)	*P* value
Control[a]	7	0	0	–	–	–	–
Control[b]	9	0	0	–	–	–	–
15[a]	4	1	0	32.0	0.1	37.8	0.1
16[a]	4	1	0	12.0	0.7	5.4	0.7
17[a]	4	1	0	21.3	0.4	35.1	0.3
18[b]	4	0	0	16.4	0.9	11.9	0.7

TWR, total worm burden reduction; FWBR, female worm burden reduction.

[a] Versus control.

[b] Versus control.

In vitro activity of non-peroxidic analogues

To elucidate the role and necessity of the peroxide core of the alkoxydioxolanes for trematocidal activity we tested the *in vitro* activity of two alkoxy-substituted tetrahydrofurans (19, 20) prepared as non-peroxidic analogues of the alkoxydioxolanes. Both derivatives lacked activity at concentrations of 30 and 90 mg/L

Table 4. *In vitro* mortality of *E. caproni* worms at timepoints 24, 48 and 72 h post-drug treatment with selected compounds

Drug	Drug concentration (mg/L)	No. of worms observed	Percentage of worms that died after indicated time			
			0 h	24 h	48 h	72 h
Control	–	20	0	0	0	0
1	100	8	0	100		
	50	8	0	100		
	10	7	0	43	100	
	5	8	0	25	75	88
2	100	6	0	100		
	50	6	0	0	100	
	10	6	0	17	67	100
	5	7	0	0	29	57
3	100	6	0	100		
	50	7	0	100		
	10	7	0	57	100	
	5	9	0	22	100	
15	100	6	0	100		
	50	6	0	100		
	10	7	0	86	100	
	5	8	0	0	38	75
16	100	6	0	100		
	50	6	0	100		
	10	7	0	100		
	5	6	0	50	67	100
17	100	6	0	100		
	50	7	0	100		
	10	6	0	100		
	5	7	0	0	100	
18	100	6	0	100		
	50	7	0	100		
	10	6	0	67	83	100
	5	6	0	0	17	33

Table 5. *In vivo* activity following a single oral dose of 400 mg/kg of selected 3-alkoxy-1,2-dioxolanes against *E. caproni* in mice

Group	No. of mice investigated	No. of mice cured	Worms	TWR (%)	*P* value
Control	4	0	23.0 (0)	–	–
15	4	0	19.0 (7.2)	17.4	0.2
16	4	0	27.8 (4.5)	0	0.2
17	4	0	23.0 (7.8)	0	0.5

TWR, total worm burden reduction.

against *S. mansoni*. Also, *E. caproni* was not affected at 50 and 100 mg/L. No effect against adult *S. mansoni* could be observed by microscopic examination 72 h post-treatment as well as by IMC 6 days after treatment. According to IMC, no loss of motility was detected for either non-peroxidic compound over an incubation period of 6 days (concentration 30 mg/L). For comparison, treatment of adult *S. mansoni* with 30 mg/L of the peroxidic analogue of compound 19 (compound 16) revealed a reduction in heat flow and complete loss of motility was detected 90 h post-exposure (Figure 2).

Discussion

The antischistosomal activity of semisynthetic artemisinins, frequently used in malaria treatment, synthetic trioxolanes and hybrid molecules of quinines and trioxanes has been well described.[7,8] Compounds characterized by a peroxidic skeleton are therefore an interesting starting point for antischistosomal drug discovery. Hence, we were interested in elucidating the antischistosomal potential of recently introduced alkoxydioxolanes as well as their structural needs for activity against schistosomes.[10]

Three of 14 compounds tested (compounds 1–3) showed promising *in vitro* activity against adult *S. mansoni* flukes. Two of these compounds (2 and 3) also revealed very good efficacy against the juvenile stage (NTS) *in vitro*. The antischistosomal *in vitro* activity seems to be enhanced by the presence of a bulky substituent at C3, since the three lead compounds all feature a large alkoxide side chain at C3. The remaining non-active 11 compounds do not display this feature. This finding is in accordance with observations reported by Schiaffo *et al.*[10] on the antimalarial structure–activity relationship of similar compounds. These studies revealed that antimalarial activity is enhanced by a spirocyclohexyl group at C5/C5′ and by the presence of a steric bulk at C3. However, for antischistosomal activity it does not seem to matter whether substitution at C5/C5′ is a dimethyl (compound 2) or spirocyclohexyl (compound 3) group. On the other hand, it is interesting to note that, with regard to *E. caproni*, the three most active compounds (3, 16 and 17) all display spirocyclohexyl units.

In our *in vivo* studies we observed only moderate, non-significant activities of alkoxydioxolanes in *S. mansoni*-infected NMRI mice. Compound 1 showed the highest *in vivo* activity against adult *S. mansoni*, achieving a total WBR of 42.5% and a female WBR of 47.1%. In contrast to results reported for artemisinins, surprisingly low WBRs were observed against juvenile *S. mansoni* harboured in mice.[18] A recent study conducted with a library of dioxolanes revealed that the majority of the compounds tested against rat and human microsomes were metabolized rapidly. It was concluded that significant optimization of the groups attached to the dioxolane core is needed before a viable candidate for drug development could be identified.[19] Hence, it is likely that the alkoxydioxolanes tested in this study also exhibit bioavailability problems which can likely play a role in limiting *in vivo* activities.

In the present work we also studied the activity of alkoxydioxolanes in the presence of various additional iron sources, since haemoglobin metabolism, the potential target of these drugs, is a common feature of both *Schistosoma* and *Plasmodium*

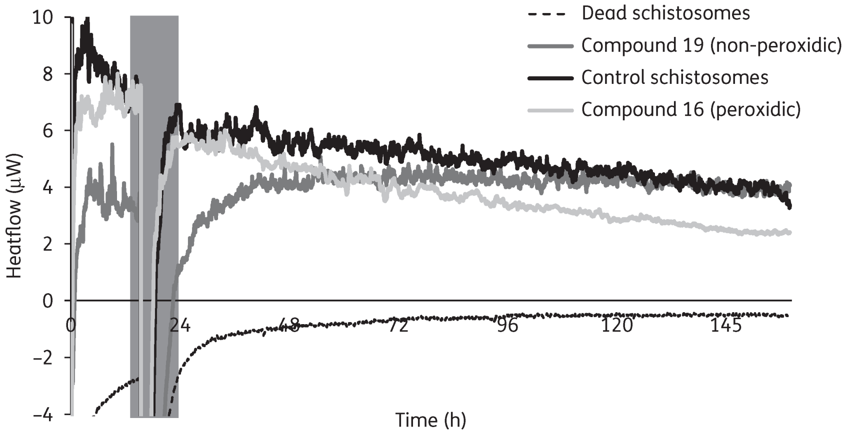


Figure 2. Course of heat flow (µW) over time (h) after treatment of schistosomes with compound 19 (non-peroxidic) or compound 16 (peroxidic). Control schistosomes correspond to schistosomes treated with DMSO at the same concentration as used for drugs (0.3%). Amplitudes of curves represent the motility of schistosomes.

spp.[20] Earlier studies investigated the relationship between Fe(II) reactivity, the efficiency of haem alkylation and antimalarial activity for various peroxides *in vitro*. The authors stated that Fe(II) reactivity for the tested peroxide heterocycles is a necessary, but insufficient, property of antimalarial peroxides,[21] and the alkoxydioxolanes have been shown to undergo cleavage to alkoxy radicals in the presence of iron(II).[10] Interestingly, we did not observe significant differences in susceptibility of the tested agents in the different iron-source-containing media. This finding is in contrast to the haemin-dependent antischistosomal *in vitro* effect of the trioxolane OZ78 on *S. mansoni* and *S. japonicum*, whereas similar haemin-independent activity was described for OZ209.[6,22] Similarly, it was recently shown that the antimalarial arylmethanol mefloquine, a drug class also described to interfere with haemoglobin degradation in *Plasmodium*, revealed a 57-fold lower IC_{50} in the presence of haemoglobin against adult *S. mansoni in vitro*.[23] Our results suggest that the alkoxydioxolanes possess an iron-independent mechanism of action on schistosomes *in vitro*. This discovery is supported by the observed high *in vitro* activities of three compounds (3, 16 and 17) at concentrations as low as 5 mg/L against the non-haematophagous intestinal fluke *E. caproni*.

Nonetheless, the results with the isosteric compounds that lack a peroxide functional group underline the necessity of the peroxidic core for trematocidal activity. Both compounds lacked *in vitro* activity against adult *S. mansoni* and *E. caproni*. Similar results were recently demonstrated in studies with the liver fluke *Fasciola hepatica*. While OZ78 has excellent *in vitro* and *in vivo* activity against *F. hepatica*, its non-peroxidic analogue failed to show an effect against the fluke.[24] The peroxidic feature seems therefore to play a role in the iron-independent mode of action. The basis for the iron-independent activity of the alkoxydioxolanes is unclear. These molecules, like all peroxides, are oxidants, and in principle capable of reaction with strongly nucleophilic or reducing agents. Alternatively, it is possible that the alkoxydioxolanes undergo activation via acid-catalysed ring opening of the peroxyacetal core to generate a more reactive 3-hydroperoxyketone; a similar model has been proposed to account for the antimalarial activity of artemisinin.[25]

In conclusion, we have demonstrated that a number of alkoxydioxolanes are characterized by good *in vitro* antischistosomal activity and non-significant *in vivo* effects on *S. mansoni*, with compound 1 being the most promising candidate. Similarities, but also differences, exist between antimalarial and antischistosomal activity of alkoxydioxolanes. The peroxidic bond is essential to antischistosomal activity, but activation of the molecules seems to be independent of iron. The low *in vivo* activity of this drug class, which may result from limited bioavailability, represents a challenge that would need to be overcome in order to identify an antischistosomal lead candidate.

Funding

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Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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5.3 Identification of Antischistosomal Leads by Evaluating Bridged 1,2,4,5-Tetraoxanes, Alphaperoxides, and Tricyclic Monoperoxides

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Identification of Antischistosomal Leads by Evaluating Bridged 1,2,4,5-Tetraoxanes, Alphaperoxides, and Tricyclic Monoperoxides

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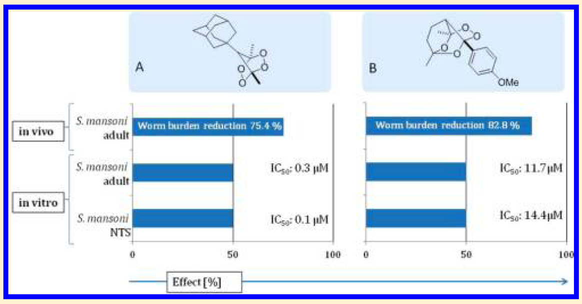
S *Supporting Information*



ABSTRACT: Although antischistosomal properties of peroxides were studied in recent years, systematic structure−activity relationships have not been conducted. We evaluated the antischistosomal potential of 64 peroxides belonging to bridged 1,2,4,5-tetraoxanes, alphaperoxides, and tricyclic monoperoxides. Thirty-nine compounds presented IC$_{50}$ values <15 μM on newly transformed schistosomula. Active drugs featured phenyl-, adamantane-, or alkyl residues at the methylene bridge. Lower susceptibility was documented on adult schistosomes, with most hit compounds being tricyclic monoperoxides (IC$_{50}$: 7.7−13.4 μM). A bridged 1,2,4,5-tetraoxane characterized by an adamantane residue showed the highest activity (IC$_{50}$: 0.3 μM) on adult *Schistosoma mansoni*. Studies with hemin and heme supplemented medium indicated that antischistosomal activation of peroxides is not necessarily triggered by iron porphyrins. Two compounds (tricyclic monoperoxide; bridged 1,2,4,5-tetraoxane) revealed high worm burden reductions in the chronic (WBR: 75.4−82.8%) but only moderate activity in the juvenile (WBR: 18.9−43.1%) *S. mansoni* mouse model. Our results might serve as starting point for the preparation and evaluation of related derivatives.

■ INTRODUCTION

Schistosomiasis remains one of the most prevalent parasitic diseases, being endemic in 76 countries worldwide with approximately 780 million people at risk of infection.[1] The infection is caused by trematodes of the genus *Schistosoma*, among which *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum* represent the most important pathogenic species for humans. During chronic infections, worms persist in the liver and hepatic portal system or urinary tract system depending on the species. Mature schistosomes start laying eggs within their habitation, which often get trapped in the tissues, resulting in inflammatory and obstructive diseases of affected organs.[2] To cure subtle morbidity and prevent the development of severe late stage morbidity, at risk populations are periodically treated with praziquantel, the drug of choice for treating schistosomiasis.[3] Following the discovery of the antischistosomal properties of the artemisinins, in recent years, various compounds characterized by a peroxidic scaffold were studied in detail for their antischistosomal activity. The overarching goal of these studies was to identify a drug with high activity against both juvenile and adult schistosomes. In contrast to praziquantel, the artemisinins revealed high activities against juvenile schistosomes but low to moderate activities on the adult worms in *S. mansoni* infected mice.[4,5] Studies with fully synthetic compounds, including ozonides,[6,7] trioxaquines,[8] and dioxolanes,[9] were undertaken. In more detail, among the aryl-ozonides, OZ418 was identified as the most promising lead candidate possessing high activity on both juvenile and adult schistosome infections in mice.[6] The trioxaquine lead candidate PA1259, a hybrid drug containing an aminoquinoline so as trioxane pharmacophore, was characterized by moderate in vivo activity on juvenile and adult *S. mansoni* in mice.[8,10] In another study, a praziquantel−ozonide hybrid was designed, which however failed to demonstrate in vivo activity.[11,12] Finally, the relationship between the peroxidic scaffold and antischistosomal activity was underscored by testing a series of alkoxydioxolanes in vitro and in vivo.[9]

However, despite the evaluation of several peroxidic compound classes for their effect on schistosomes, only little is known on the relationship between the peroxidic structures and antischistosomal activity. Therefore, we were interested in elucidating the antischistosomal potential of three practically

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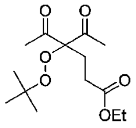
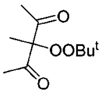
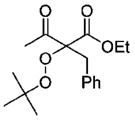
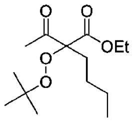
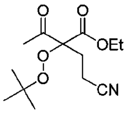
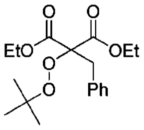
Table 1. Chemical Structures of Investigated Structures Presented Regarding Their Peroxidic Class[a]

Alphaperoxides **1-17** which are α-*tert*-butylperoxy derivatives of α-substituted	acetyl acetone [16]					
		1	**2**			
	acetoacetic ester[16]					
		3	**4**	**5**		
	malonic ester[16]					
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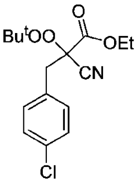
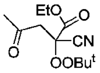
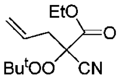
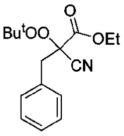
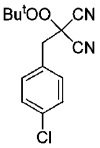
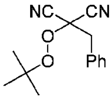
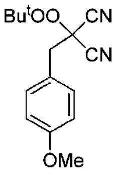
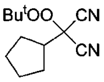
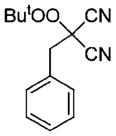
	cyanoacetic ester [15]					
		7	**8**	**9**	**10**	
	malono nitrile[15]					
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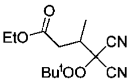
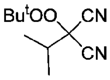
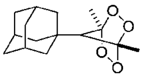
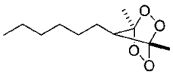
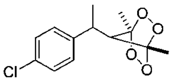
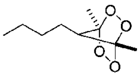
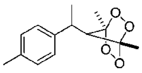
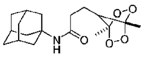
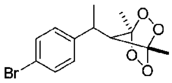
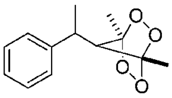
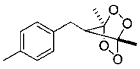
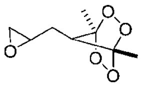
						
		16	**17**			

Table 1. continued

Bridged 1,2,4,5-tetraoxanes(*)[14] **18-44**					
	18	**19**	**20**	**21**	**22**
					
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	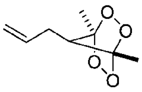	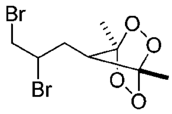	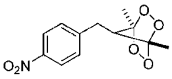	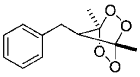	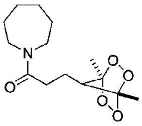
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	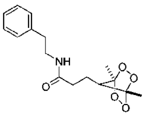	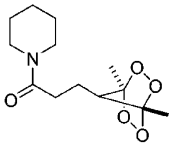	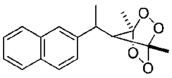	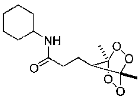	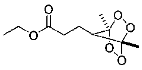
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	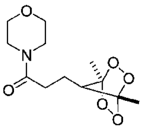	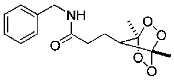	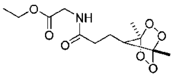	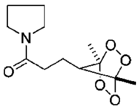	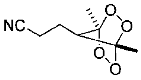
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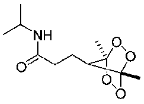
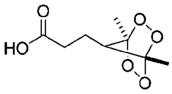
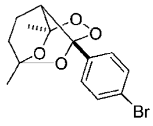
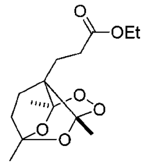
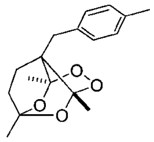
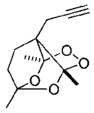
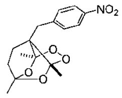
					
	43	**44**			

Table 1. continued

Tricyclic monoperoxides [17] **45-63**					
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	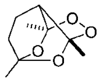	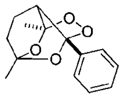	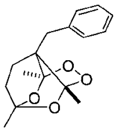	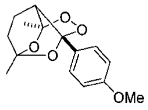	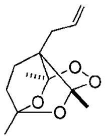
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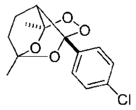
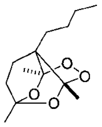
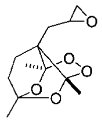
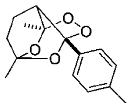
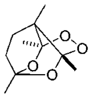
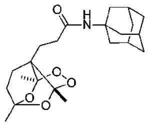
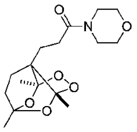
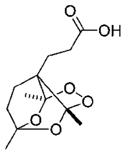
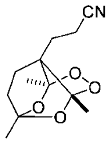
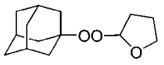
					
	55	**56**	**57**	**58**	**59**
					
	60	**61**	**62**	**63**	
2- (1- adamantylperoxy) -tetrahydrofuran (*) **64**					
	64				

[a]*Detailed information can be found in Supporting Information.

new peroxide classes, namely candidates of β-dicarbonyl compounds and their heteroanalogues, bridged 1,2,4,5-tetraoxanes and alphaperoxides so as β,δ-triketones, tricyclic monoperoxides, in vitro and in vivo against *S. mansoni* for the first time. Bridged 1,2,4,5-tetraoxanes present with a methylene bridge within the pharmacophore which distinguishes them from described 1,2,4,5-tetraoxanes with known trematocidal activity.[13] The preparation of these peroxide classes has been

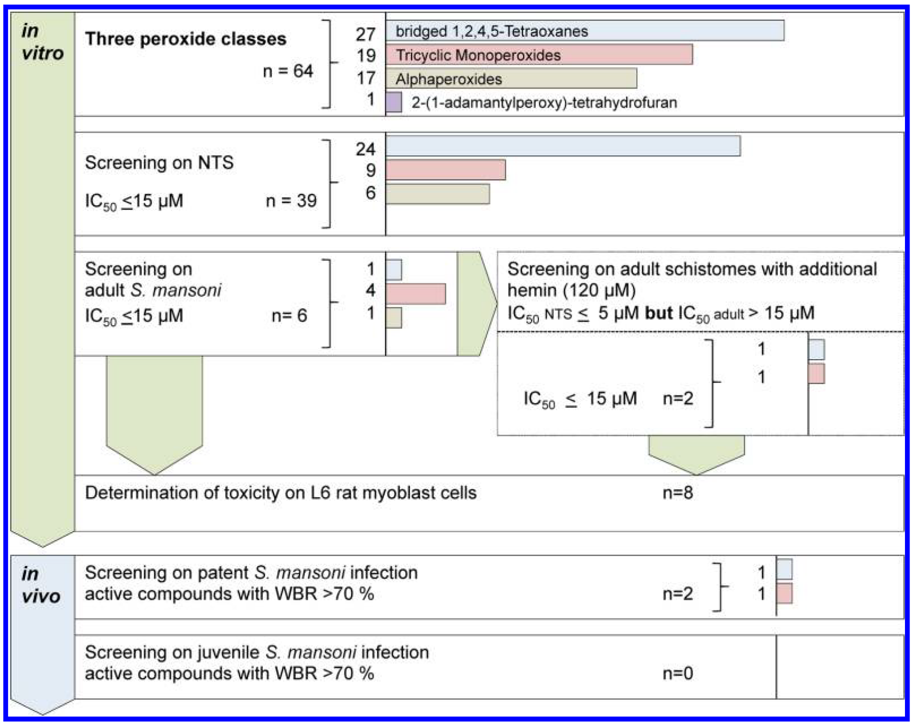


Figure 1. Flowchart of our screening procedures using three different peroxide classes and one 2-(1-adamantylperoxy)-tetrahydrofuran.

developed recently, and their syntheses imply high yields using inexpensive and accessible reagents.[14−17] A library with small druglike molecules showing high structural variability was synthesized for each class of compounds and tested in vitro on newly transformed schistosomula (NTS) and adult *S. mansoni*. The antischistosomal activity of selected compounds was additionally studied in the presence of hemin and heme, as possible activators. Compounds revealing high activity in vitro were next tested on toxicity and further characterized in vivo using a patent *S. mansoni* mouse model. Finally, compounds being active in the chronic infection model progressed into the juvenile infection model to characterize the full spectrum of activity of these peroxides.

■ RESULTS

In Vitro Screening on NTS. In a first step, the four compound libraries consisting of 27 tetraoxanes, 19 tricyclic monoperoxides, 17 alphaperoxides, and one 2-(1-adamantylperoxy)-tetrahydrofuran (Table 1) were tested on *S. mansoni* NTS. Our workflow is presented in Figure 1. Results are summarized in Table 2. Thirty-nine compounds (24 tetraoxanes, 9 tricyclic monoperoxides, and 6 alphaperoxides) showed high activities (as defined by half-maximal inhibitory concentrations (IC_{50}s) ≤ 15 μM) on NTS. In more detail, 24 out of 27 tetraoxanes (89%) revealed IC_{50} values ranging from 0.1 to 13.9 μM. Six of these had IC_{50} values even lower than 1 μM, with compounds **18** (IC_{50}: 0.1 μM) and **19** (IC_{50}: 0.2 μM) displaying the highest activity. Hence the most active tetraoxanes showed at least 10-fold increased activity when compared to reference drug praziquantel (IC_{50}: 2.2 μM) and 25-fold increase in activity when compared to artesunate (IC_{50}: 5.0 μM) on the schistosomular stage. The remaining three of the 27 tetraoxanes tested (compounds **42**−**44**) showed low to moderate activity (IC_{50}s: 15.3−58.1 μM). Compound **45** was the most active compound in the class of tricyclic monoperoxides (IC_{50}: 1.7 μM). Eight tricyclic monoperoxides had IC_{50} values ranging from 3.5 to 14.4 μM. The remaining compounds showed low to moderate activity (IC_{50}s: 15.6−213.2 μM **54**−**60**) or lacked activity (**61**−**63**) (motility decreased less than 50% at the highest concentration tested). Of the 17 alphaperoxides tested, six compounds (**6**, **7**, **9**, **13**, **14**, **17**) were highly active (IC_{50}s: 3.9−14.6 μM), eight molecules showed low to moderate activities (IC_{50}s 15.1−293.0 μM), and three compounds lacked activity (**1**, **2**, **5**). Finally, the tested 2-(1-adamantylperoxy)-tetrahydrofuran (**64**) lacked activity on the schistosomular stage.

In Vitro Screening on Adult Schistosomes. Thirty-nine compounds progressed into the adult *S. mansoni* screens (Table 2). Of these, six compounds (1 tetraoxane, 4 tricyclic monoperoxides, and 1 alphaperoxide) showed high activity (defined as IC_{50}s ≤ 15 μM) against adult *S. mansoni*. The highest activity was observed with tetraoxane **18** characterized by an IC_{50} of 0.3 μM, revealing comparable in vitro activity as the reference drug praziquantel (IC_{50}: 0.1 μM). The two tricyclic monoperoxides (**51** and **45**) presented IC_{50} values of 7.7 and 8.7 μM, respectively. Compounds **12** (alphaperoxide), **49**, and **53** (tricyclic monoperoxides) revealed similar IC_{50} values of 14.7, 12.2, and 11.7 μM, respectively.

Twenty-two compounds (16 tetraoxanes, 2 tricyclic monoperoxides, and 4 alphaperoxides) showed a low to moderate activity (IC_{50}s: 18.8−132.4 μM). Finally, of the 39 compounds tested, 11 revealed no decrease of motility of more than 50% on adult *S. mansoni* at the highest concentration tested.

All compounds which revealed a high antischistosomal potential on NTS (IC_{50} < 5.0 μM) but only low, moderate, or no activity against adult schistosomes (12 tetraoxanes, 2

Table 2. In Vitro Activity of Three Different Peroxide Classes (Alphaperoxides, Bridged 1,2,4,5-Tetraoxanes, and Tricyclic Monoperoxides) and One 2-(1-Adamantylperoxy)-tetrahydrofuran on NTS and adult *S. mansoni*[a]

		NTS *S. mansoni*		adult *S. mansoni*		adult *S. mansoni* with hemin (120 μM)	
compd	no.	IC$_{50}$ [μM]	*r*	IC$_{50}$ [μM]	*r*	IC$_{50}$ [μM]	*r*
artesunate		4.97	0.9	41.2	0.8	38.7	0.7
praziquantel		2.2	0.9	0.1	0.9		
alphaperoxides	**1**	>104					
	2	>795					
	3	15.1	0.9				
	4	26.5	0.9				
	5	>111					
	6	14.6	0.9	59.1	0.8		
	7	9.0	0.9	>126.8			
	8	37.8	0.8				
	9	43.2	0.9				
	10	67.7	1.0				
	11	3.9	0.9	19.3	0.8	17.9	0.9
	12	5.0	0.9	14.7	0.9		
	13	8.0	0.9	40.6	1.0		
	14	10.7	0.9	122.9	0.9		
	15	24.1	0.9				
	16	157.0	0.9				
	17	293.0	0.3				
bridged 1,2,4,5-tetraoxanes	**18**	0.1	0.9	0.3	1.0		
	19	0.2	1.0	61.5	0.9	27.3	0.9
	20	0.4	0.9	35.5	0.9	25.3	1.0
	21	0.5	1.0	37.8	1.0	>159	
	22	0.6	0.9	30.9	1.0	41.2	0.9
	23	0.8	0.8	33.3	1.0	39.3	1.0
	24	1.0	0.9	33.6	0.9	47.7	0.9
	25	1.1	0.9	18.8	1.0	12.9	0.9
	26	1.2	0.8	40.0	0.9	>127	
	27	1.2	0.8	132.4	0.9	91.9	0.9
	28	3.4	0.9	49.8	0.9	>174	
	29	3.4	0.9	>89.8		>89.8	
	30	4.4	1.0	>112		>112	
	31	5.0	0.9	41.4	0.7	134.9	0.9
	32	5.5	0.9	50.1	0.9		
	33	6.1	1.0	65.6	0.9		
	34	6.6	1.0	>110.6			
	35	7.1	0.8	35.5	1.0		
	36	8.9	0.9	66.6	0.9		
	37	9.8	0.9	>129.2			
	38	10.7	0.9	36.9	0.8		
	39	10.9	0.9	>102			
	40	11.7	0.9	>103.7			
	41	13.9	0.9	>117			
	42	15.3	1.0				
	43	25.6	0.9				
	44	58.1	0.8				
tricyclic monoperoxides	**45**	1.7	0.8	8.7	0.9		
	46	3.5	0.9	>105		92.2	0.9
	47	4.5	0.9	>103		13.4	0.9
	48	8.1	1.0	82.4	0.9		
	49	9.0	0.9	12.2	0.8		
	50	9.0	1.0	19.2	0.9		
	51	9.3	0.8	7.7	0.9		
	52	13.4	0.9	>109			
	53	14.4	0.8	11.7	0.9		

Table 2. continued

		NTS *S. mansoni*		adult *S. mansoni*		adult *S. mansoni* with hemin (120 μM)	
compd	no.	IC_{50} [μM]	*r*	IC_{50} [μM]	*r*	IC_{50} [μM]	*r*
	54	15.6	0.9				
	55	16.0	0.9				
	56	22.3	0.9				
	57	22.5	0.8				
	58	45.3	0.9				
	59	180.4	0.9				
	60	213.2	0.9				
	61	>287					
	62	>348					
	63	>376					
2-(1-adamantylperoxy)-tetrahydrofuran	**64**	>125.9					

[a] R represents the "goodness of fit," whereas >0.85 is acceptable.

tricyclic monoperoxides, and 1 alphaperoxide) were retested in the presence of Fe(III)-hemin. In addition, four of these compounds were also studied in a Fe(II)−heme supplemented media.

Selected compounds of alphaperoxides (**11**) and tricyclic monoperoxides (**46**) did not show a great difference in their IC_{50} values when incubated with (**46**, 92.2 μM; **11**, 17.9 μM) or without (**46**, >123 μM; **11**, 19.3 μM) hemin. Similar patterns were also observed for the 13 tested tetraoxanes. The majority (9 compounds: **19**, **20**, **22**−**25**, **27**, **29**, **30**) showed comparable antischistosomal activity with or without hemin or slightly decreased activity (**21**, **26**, **28**, and **31**). A considerable increase of activity was detected for one of the selected tricyclic monoperoxides, compound **47**, lacking activity (>103 μM) without and showing an IC_{50} value of 14.4 μM with additional hemin. Finally, in the presence of a Fe(II)−heme supplemented media, three compounds still lacked activity (**30**, **46**, **47**) and only one showed a slight increase in activity (**29**; IC_{50}: 33.6 μM).

Determination of Cytotoxicity. The eight compounds displaying high activity against adult *S. mansoni* in vitro were first tested on L6 cells for their cytotoxic potential. Artesunate and praziquantel were used as reference drugs, and data are summarized in Table 3. The two tricyclic monoperoxides (**47**, **49**) and praziquantel showed no cytotoxic potential at the highest concentration tested (30 μg/mL). Lowest cytotoxic potential with IC_{50} values of 94.4 and 58.3 μM were observed for the alphaperoxide **12** and the tricyclic monoperoxide **53**, respectively. The tricyclic monoperoxides, **45** and **51**, revealed similar moderately cytotoxic IC_{50} values from 8.2 μM (**46**) to 10.3 μM (**52**). Furthermore, both selected tetraoxanes **18** (1.7 μM) and **25** (2.2 μM) showed similar cytotoxic effects on L6 cells as observed for artesunate (IC_{50}: 1.5 μM). Selectivity indices ranged from 0.2 for **25** to 7.6 for **49** (Table 3). Lead candidates (**18**, **53**) (based on the in vivo activity results, see below) and artesunate were further investigated on two different human cell lines, namely HeLa and MRC-5. All compounds showed 2-fold higher effects on the cancer cell line (HeLa) than on the normal cell line (MRC-5). The lowest cytotoxicity was determined for the tricyclic monoperoxide **53** (IC_{50} of 6.0 μM on HeLa and 12.4 μM on MRC-5) followed by artesunate (IC_{50} of 3.2 μM on HeLa and 6.8 μM on MRC-5). Tetraoxane **18** showed cytotoxic potential on both cell lines (IC_{50} of 0.4 μM on HeLa and 1.2 μM on MRC-5).

Table 3. IC_{50} Values of Eight Selected Hit Compounds (12, 18, 25, 45, 47, 49, 51, and 53) Evaluated with L6 Cells and Adult *S. mansoni* Worms[a]

	IC_{50} [μM]		
compd	L6 cells (SD)	*S. mansoni*	SI
12	99.0 (11.0)	14.7	6.4
18	1.7 (0.3)	0.3	5.7
25	2.2 (0.4)	18.8	0.1
45	8.2 (1.9)	8.7	0.9
47	>103	13.4	>7.7
49	>93	12.2	>7.6
51	10.3 (1.8)	7.7	1.3
53	58.3 (17.9)	11.8	4.9
artesunate	1.5 (0.6)	38.7	0.04
praziquantel	>96	0.1	>960

[a] SD: standard deviation. SI: selectivity index. Selectivity indices (SI) were calculated based on evaluated IC_{50} values and artesunate and praziquantel served as control compounds.

In Vivo Efficacy on Patent *S. mansoni* Infection. On the basis of their in vitro activity against adult schistosomes (IC_{50} < 15 μM), two tetraoxanes (**18**, **25**), five tricyclic monoperoxides (**45**, **47**, **49**, **51**, and **53**), and one alphaperoxide (**12**) were tested in mice harboring a patent *S. mansoni* infection (Table 4). The alphaperoxide **12** and tricyclic monoperoxide **47** lacked in vivo activity. Treatment with three of the tricyclic monoperoxides and one of the tetraoxanes resulted in low total worm burden reductions (WBRs) ranging from 4.7 to 31.3% (**25**, 5.0%; **45**, 31.3%; **49**, 4.7%; **51**, 6.5%). Good antischistosomal in vivo activity was observed with tetraoxane **18** and tricyclic monoperoxide **53**. Compound **18** achieved total and female WBRs of 75.4% (p = 0.03) and 77.8% (p = 0.03), respectively. For the tricyclic monoperoxide **53**, significant total and female WBRs of 82.8% (p = 0.02) and 82.9% (p = 0.01), respectively, were determined.

In Vivo Efficacy Studies on Juvenile *S.mansoni* Infection. Compounds **18** and **53** were tested against juvenile *S. mansoni* in vivo. The tetraoxane showed moderate total and female WBRs of 43.1% and 50%, respectively. Low WBRs were observed with the tricyclic monoperoxide with 18.9% total and 27.3% female WBRs.

Table 4. In Vivo Activity of Selected Compounds from Three Different Peroxide Classes (Bridged 1,2,4,5-Tetraoxanes, Tricyclic Monoperoxides, Alphaperoxides)[a]

		mean number of worms (SD)		adult infection [%]		juvenile infection [%]	
compd (chemical class)	no. mice investigated	total	females	TWR	FWBR	TWR	FWBR
control[a]	9	29.0 (28.5)	13.3 (12.9)				
control[b]	8	38.3 (18.1)	20.6 (8.8)				
control[c]	10	30.6 (24.7)	16.1 (13)				
control[d]	8	21.6 (12.2)	11.0 (6.4)				
12[a]	4	32.3 (21.4)	17.3 (11.6)	0	0		
18[a]	6	6.7 (2.5)	2.3 (1.2)	75.4*	77.8*	43.1	50.0
25[d]	2	20.5 (11.5)	10.5 (9.2)	5.0	4.6		
45[c]	4	21.0 (2.2)	10.3 (3.1)	31.3	36.4		
47[d]	4	22.3 (4.9)	12.0 (2.4)	0	0		
49[d]	4	23.5 (5.9)	11.8 (3.1)	4.7	9.6		
51[b]	4	35.8 (8.2)	17.5 (6.6)	6.5	15.2		
53[c]	4	5.3 (5)	2.8 (2.6)	82.8*	82.9*	18.9	27.3

[a] All tested on patent schistosoma infection (49 days post treatment) and promising candidates as well on juvenile schistosoma infections (21 days post-treatment). TWR: total worm burden reduction. FWBR: female worm burden reduction. SD: standard deviation. *p-value < 0.05 using KW test.

■ DISCUSSION AND CONCLUSION

In recent years, peroxides have played a prominent role in antischistosomal drug discovery and development. Various studies have been conducted ranging from preclinical in vitro and in vivo studies as well as clinical trials.[4,8] Nonetheless, to date, our knowledge is still limited with regard to the structural requirements these molecules need in order to elicit antischistosomal activity. Therefore, in the present work, three different peroxide classes were screened for their antischistosomal potential.

First, compounds were studied for activity against NTS (Figure 1). Compounds with an activity <15 μM against NTS were classified as active and progressed further. Abdulla and colleagues recently described a similar screening workflow, however, using a 15-fold lower cutoff of 1 μM to obtain an acceptable hit rate of 10%.[18] On the other hand, Mansour and Bickle noted that schistosome active drugs were best identified in their screen using concentrations of 10 μg/mL (i.e., 28−44 μM) in the primary NTS screen.[19] Given the excellent activity of peroxidic drugs against juvenile schistosomes,[4,6] as mentioned in this work, an IC_{50} value <15 μM was selected as cutoff.

Of 64 compounds tested, 39 (60%) showed high in vitro activity against NTS. For the class of tetraoxanes, we detected the highest activity against the schistosomular stage (with 89% of compounds being active), whereas within the other two peroxide classes investigated (tricyclic monoperoxides and alphaperoxides), less than half of the compounds displayed activity. Most of the active tetraoxanes elucidated activities comparable to praziquantel on this parasite stage. Structural variation was observed among active compounds on schistosomula. However, a tendency of structural features among highly active tetraoxanes and tricyclic monoperoxides could be noted. All highly active structures among these two groups presented either phenyl- (**20**, **22**, **24**−**26**, **30**, **31**, and **45**), adamantane- (**18**, **23**), or alkyl- (**19**, **21**, and **28**) residues at the methylene bridge.

In contrast to results obtained on NTS with nearly all tetraoxanes being active, adult *S. mansoni* showed a lower susceptibility to these drugs with only one tetraoxane (**18**) revealing prominent activity. This compound revealed also high worm burden reductions against *S. mansoni* in vivo. Compound **18** displays an adamantane substitute at the methylene bridge. Early studies with synthetic peroxides in the framework of a collaborative antimalarial discovery project evaluated essential characteristics for a new trioxolane antimalarial drug. It was documented that necessary pharmacokinetic characteristics could be obtained with the spiroadamantane trioxolane pharmacophore.[20] Increased lipophilicity of the adamantane substituted compounds resulted in higher antimalarial activity. In addition, using the same class of compounds as a starting point to search for a fasciocidal synthetic peroxide drug development candidate revealed that the spiroadamantane substructure is an essential part for fasciocidal activity.[21] Contrary to the known active spiroadamantane substructures, compound **18** does not contain a spiro-fragment; the adamantane and tetraoxane parts are joined directly with a C−C bond.

It is interesting to note that the tested 2-(1-adamantylperoxy)-tetrahydrofuran (**64**) did not expose any activity on the schistosomular stage which indicates that not only the presence of an adamantane part determines the antischistosomal activity. Therefore, most probably, the key fragment which determines activity is the bridged tetraoxane and adamantane bears a supporting function.

The class of tricyclic monoperoxides revealed the greatest number of hits (n = 4) on adult *S. mansoni* in vitro, with compounds **45** and **51** being most active. Three of the active tricyclic monoperoxides (**45**, **51**, and **53**) have a phenyl residue next to the peroxidic bond in common. To note, these phenyl-containing peroxides are unusual compounds from the chemical point of view; generally peroxides containing the Ar−C−O−O moiety easily decompose in accordance with heterolytic mechanism by Hock and related reactions.[22,23]

Furthermore, the substitution in the bridge of tricyclic monoperoxides seems to affect the activity as observed on the schistosomular stage likewise. Phenylic residues (as seen for **47**) seem to increase the activity, whereas a propargyl substituent results in decreased activity (as seen for **48**). However, among the three tested compounds (**45**, **51**, and **53**) in vivo, only **53** achieved a promising WBR of 82.8%. This might be explained with a higher metabolic stability of the methylether substituted compound.[24]

It is interesting to note that, in general, adult *S. mansoni* were less affected by the peroxides than NTS. This was particularly striking for the alphaperoxides and as mentioned before for the bridged 1,2,4,5-tetraoxanes. While six alphaperoxides showed a high activity against NTS, only one was active against the adult worms. In a recent study using a random collection of 33 compounds with proven in vitro activity on adult schistosomes and 30 compounds with lacking adult activity, none of the compounds lacking activity on adult worms revealed significant activity on NTS.[19] This finding suggests a superior susceptibility of schistosomula to peroxidic compounds.

Since it was previously proposed that hemin increases the activity of peroxidic structures, as demonstrated for artemether,[25,26] additional experiments were conducted using hemin (Fe(III)) as well as heme (Fe(II)) in the incubation medium. Interestingly, only one tricyclic monoperoxide (**47**) showed increased in vitro activity in the presence of hemin, likewise, there was only one compound (**29**) which elucidated a slightly increased activity in the presence of heme. This moderately increased in vitro activity could be explained by an additional activation of the drug within the medium and not only within the parasites gut which was proposed as possible interaction site of hemin and artemether for *S. japonicum*.[26] Most of the selected tetraoxanes showed similar to decreased efficacy when incubated with hemin or heme, and no changes on the motility were observed in the supplemented media for the selected alphaperoxides. Note that slight fluctuations in IC_{50} values based on microscopical readout might be due to differences in sensitivities of worms or the subjective readout used. Nonetheless, these results indicate that an antischistosomal activation of peroxides is not necessarily triggered by hemin or heme or at least does not represent the only activator because great variations were not observed for the two tested peroxide classes (alphaperoxides and 1,2,4,5-tetraoxanes) in the different media.

Only low to moderate activity was observed for the two hit candidates, tetraoxane **18** (WBR: 43.1%) and the tricyclic monoperoxide **53** (WBR: 29.1%) against juvenile *S. mansoni* infections in mice, which is in contrary to the recently investigated ozonides or the artemisinins.[5,6] Hence, the activity profile of the investigated peroxides is different from previously studied peroxidic compounds, a finding which cannot be explained at the moment.

Interestingly, a high cytotoxic potential was observed for artesunate on all tested celllines, which is in accordance to recently shown induction of cell death by artemisinin compounds and cytotoxic observations on HepG2 cells.[27] The activity of peroxides on blood-feeding parasites is most probably dependent on the activation of the endoperoxide bridge by an iron(II) species leading to C-centered radicals, which might be responsible for cytotoxicity.[27−29] Hence, it is not surprising that some of the tested peroxides showed a higher cytotoxic potential on L6 cells than the nonperoxidic reference drug praziquantel. Furthermore, it is known that the artemisinins possess cytotoxic potential on various cancer cell lines,[30] and apoptopic processes of fast proliferating cells in prescence of iron have been described.[27] It is worthwhile stating that the class of alphaperoxides did not show cyctotoxic potential at the highest concentration tested. Most of the tricyclic monoperoxides showed none to moderate cytotoxicity, whereas the class of tetraoxanes showed a similar cytotoxic potential as artesunate. With regard to our lead compounds (**18** and **53**), the conducted in vitro cytotoxicity assay on L6 cells showed that both lead structures presented adequate selectivity indices when compared to artesunate. However, compound **18** elucidated an increased cytotoxic potential compared to artesunate on tested human cell lines, which has to be kept in mind as potential drawback.

In conclusion, the screening of three peroxides classes identified two interesting hit compounds, tetraoxane **18** and the tricyclic monoperoxide **53**, which both revealed a high activity against adult *S. mansoni* in vivo. On the other hand, no promising activity was detected within the class of alphaperoxides. Our results hint to the fact that an adamantane group represents an important feature for antischistosomal activity. Compounds **18** and **53** might serve as starting candidates for further lead modifications aiming to increase activity on juvenile schistosomes and to lower cytotoxic potential.

■ EXPERIMENTAL SECTION

Drugs and Media. The 63 (**1**−**63**) compounds belonging to three types of peroxide classes (bridged 1,2,4,5-tetraoxanes, tricyclic monoperoxides, and alphaperoxides) illustrated in Table 1 were prepared based upon methods described by Terent'ev and colleagues.[14−17] Additionally, a 2-(1-adamantylperoxy)-tetrahydrofuran (**64**) was prepared from 1-adamantylhydroperoxide and 2,3-dihydrofuran as described in the Supporting Information. 1-Adamantylhydroperoxide was prepared from 1,3-dehydroadamantane and H_2O_2 in accordance with Son and colleagues.[31] A hemin solution (1.5 mM) was prepared as follows: 50 mg of hemin-chloride (Fluka Analytical, Netherlands) was dissolved in 10 mL of 0.1 M NaOH and 39.5 mL of PBS (pH = 7.4). A Fe(II) heme solution (1.5 mM) was prepared by addition of 5 mM dithionite (Sigma Aldrich) to the prepared hemin solution, adapted from Barr et al.[32] Praziquantel and artesunate were purchased from Sigma-Aldrich GmbH.

Synthesis and Analytical Data for Key Compounds. ***7-(1-Adamantyl)-1,4-dimethyl-2,3,5,6-tetraoxabicyclo[2.2.1]heptane 18.***[13] A 37% aqueous H_2O_2 solution (0.353 g, 3.84 mmol) was added to a solution of 3-(1-adamantyl)pentane-2,4-dione (0.3 g, 1.28 mmol) in EtOH (3 mL), the reaction mixture was cooled to 10 °C, and a solution of H_2SO_4 (2 g, 0.02 mol) in EtOH (2 mL) was added with stirring. The reaction mixture was stirred at 20−25 °C for 1 h. Then CH_2Cl_2 (30 mL) was added. The organic layer was washed with water (2 × 10 mL), a 5% aqueous $NaHCO_3$ solution (2 × 10 mL), and again with water (2 × 10 mL), dried with Na_2SO_4, and filtered. The solvent was removed using a water-jet vacuum pump. Product 7-(1-adamantyl)-1,4-dimethyl-2,3,5,6-tetraoxabicyclo[2.2.1]heptane **18** was isolated by silica gel chromatography with elution by a hexane−ethyl acetate (EA) mixture using the gradient of the latter from 0 to 30%. Product **18** was obtained in 68% yield (0.231 g, 0.87 mmol). White crystals; mp = 130−131 °C (partially decomposed); $R_f = 0.60$ (TLC, hexane−EA, 5:1). ^1H NMR (300.13 MHz, $CDCl_3$), δ: 1.63−2.04 (m, 21H), 2.37 (s, 1H). ^{13}C NMR (75.48 MHz, $CDCl_3$), δ: 12.7, 28.3, 33.0, 36.7, 40.6, 66.8, 110.6. Anal. Calcd for $C_{15}H_{22}O_4$: C, 67.64; H, 8.33. Found: 67.37; H, 8.61.

3-(4-Methoxyphenyl)-6,7a-dimethyltetrahydro-3H,4H-3,6-epoxy-[1,2]dioxolo[3,4-b]pyran 53.[16] A 37% aqueous H_2O_2 solution (0.158 g, 1.72 mmol) and a solution of H_2SO_4 (1.0 g, 0.01 mol) in EtOH (1 mL) were added with stirring to a solution of 3-(4-methoxybenzoyl)-heptane-2,6-dione (0.30 g, 1.14 mmol) in EtOH (4 mL) at 10−15 °C. The reaction mixture was stirred at 20−25 °C for 1 h, and a mixture of CH_2Cl_2− hexane = 1:1 (10 mL) was added. Then $NaHCO_3$ was added to the reaction mixture with stirring until the pH reached 7.0. The precipitate was filtered off. The filtrate was dried over Na_2SO_4, the precipitate was filtered off, and the solvent was removed in a water jet vacuum. Product 3-(4-methoxyphenyl)-6,7a-dimethyltetrahydro-3*H*,4*H*-3,6-epoxy[1,2]dioxolo[3,4-*b*]pyran **53** was isolated by chromatography on SiO_2 using a hexane−ethyl acetate mixture as the eluent with a gradient of ethyl acetate from 5 to 50 vol %. Product **53** was obtained in 41% yield (0.131 g, 0.47 mmol). White crystals; mp = 89−90 °C; $R_f = 0.52$ (TLC, hexane−EA, 2:1). ^1H NMR (300.13 MHz,

$CDCl_3$): δ 1.55 (s, 3H), 1.61 (s, 3H), 1.68−1.80 (m, 4H), 2.62−2.66 (m, H), 3.80 (s, 3H), 6.90 (d, 2H, *J* = 8.8 Hz), 7.49 (d, 2H, *J* = 8.8 Hz). ^{13}C NMR (75.48 MHz, $CDCl_3$): δ 12.5, 17.9, 24.8, 29.3, 50.5, 55.3, 95.8, 105.6, 106.5, 113.9, 124.7, 128.1, 160.5. Anal. Calcd for $C_{15}H_{18}O_5$: C, 64.74; H, 6.52. Found: C, 64.73; H, 6.75. HRMS (ESI) *m*/*z* $[M + H]^+$ calcd for $[C_{15}H_{19}O_5]^+$ 279.1227; found 279.1227.

Instrumentation and Methods. NMR spectra of novel compounds were recorded on a commercial instrument (300.13 MHz for ^1H, 75.48 MHz for ^{13}C) in $CDCl_3$. The TLC analysis was carried out on standard silica gel chromatography plates. The melting points were determined on a Kofler hot-stage apparatus. Chromatography was performed on silica gel (63−200 mesh). Elemental analysis on carbon, hydrogen, and nitrogen was carried out using a 2400 Perkin-Elmer CHN analyzer. Determination of purity of all peroxides was executed by elemental (combustion) analysis. For all peroxides, deviation from the theoretical values for C, H, and N content was less than 0.4%. These data confirm >95% purity of compounds **1**−**64**. Structures of all compounds were confirmed using ^1H and ^{13}C NMR spectra.

Maintenance of Mice and Infection with *S. mansoni*. The in vivo studies were approved by the veterinary authorities of the Canton Basel-Stadt. Female NMRI mice (3-week old, weight ca. 14 g) were purchased from Charles River (Sulzfeld, Germany) or Harlan Laboratories (Horst, The Netherlands). Prior to infection, animals were allowed to adapt for one week under controlled conditions (temperature ca. 22 °C, humidity ca. 50%, 12 h light and 12 h dark cycle, free access to rodent diet and water). Mice were infected with *S. mansoni* (Liberian strain) by subcutaneous injection of ∼100 cercariae. Cercariae were harvested from infected intermediate host snails *Biomphalaria glabrata* by exposure to light for 3 h, following standard procedures of our laboratory.

In Vitro Compound Screening on *S. mansoni* NTS. Harvested *S. mansoni* cercariae were mechanically transformed to NTS following standard procedures.[33,34] The obtained NTS suspension was adjusted to a concentration of 100 NTS per 50 μL using Medium 199 (Invitrogen, Carlsbad, CA) supplemented with 5% heat-inactivated fetal calf serum (iFCS), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA). NTS suspensions were incubated (37 °C, 5% CO_2 in ambient air) for a minimum of 12−24 h until usage to ensure completed conversion into schistosomula.[35] On the following day, drug dilution series were prepared in 96-flat bottom well-plates (BD Falcon, USA) with concentrations ranging from 0.37 to 90 μg/mL (0.37, 1.1, 3.3, 10, 30, 90 μg/mL) using supplemented (iFCS and antibiotics) Medium 199. The prepared NTS suspension was then added to each well, and plates were incubated at 37 °C, 5% CO_2. NTS incubated in the presence of the highest DMSO concentration served as control. NTS were evaluated by microscopical readout (Carl Zeiss, Germany, magnification 80×) with regard to death, changes in motility, viability, and morphological alterations 72 h post drug exposure. Drug effects were evaluated using a viability scale as described recently.[33,34] Each concentration was tested in duplicate, and experiments were performed at least three times. IC_{50} values of test compounds were determined as described before.[36] Compounds were defined as highly active with IC_{50} values ≤15 μM, moderate activity was defined as IC_{50} 16−40 μM, and low activity for values >40 μM.

In Vitro Compound Screening on Adult *S. mansoni*. Highly active compounds ($IC_{50} \leq 15$ μM) on NTS were studied on adult schistosomes (workflow presented in Figure 1). Adult flukes were harvested from the hepatic portal veins and mesenteric veins of infected NMRI mice (7−8 weeks post infection) following standard procedures.[7] Schistosomes were placed in RPMI 1640 culture medium supplemented with 5% iFCS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C, 5% CO_2 until usage. Supplemented RPMI 1640 medium and drug stock solutions (10 mg/mL) were used to obtain final test concentrations of 1.1−30 μg/mL (1.1, 3.3, 10, 30 μg/mL) in 24-flat bottom well-plates (BD Falcon, USA) with a final volume of 2 mL. At least three schistosomes of both sexes were next added to each well. Schistosomes incubated in the presence of blank medium supplemented with the highest concentration of DMSO used in the assay served as control. Then 24, 48, and 72 h post drug exposure, schistosomes were examined phenotypically using the motility scale described before[37] and an inverse microscope (Carl Zeiss, Germany, magnification 80×). Experiments were repeated at least three times and IC_{50} values determined.[36]

The IC_{50} determination (72 h post drug exposure) for selected compounds was repeated with the addition of hemin (120 μM). Compounds showing high antischistosomal potential on the schistosomular stage ($IC_{50} \leq 5$ μM) but only moderate, low, or no activity on adult schistosomes (without hemin supplementation) were selected for these additional experiments. Experiments were performed and repeated as described above with exception of hemin supplementation (120 μM) during the entire drug exposure time. Compounds which showed very good activities on NTS ($IC_{50} \leq 5$ μM) and lacked activity on adult worms were furthermore tested in Fe(II)-heme (120 μM) supplemented media.

Determination of Cytotoxicity. The determination of cytotoxicity was performed with L-6 cells according to a previously reported procedure.[38] Briefly, L-6 cells were seeded in 96-well microtiter plates at a density of 4×10^4 cells/mL in RPMI 1640 medium with 10% fetal bovine serum and L-glutamine (2 mM). Drugs serially diluted 3-fold ranging from 0.123 to 30 μg/mL in test medium were added. The plates were incubated at 37 °C at an atmosphere of 5% CO_2. After 70 h, Alamar Blue (10 μL) was added to each well, and incubation was continued for another 2 h. The plate was then read using a SpectraMax M2 (Molecular Devices) instrument by use of an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Fluorescence development was expressed as percentage of the control, and the IC_{50} values were determined. Experiments were performed at least three times and IC_{50} values calculated as averages. The selectivity indices (SI) of tested compounds were calculated by dividing IC_{50} values obtained on L6 cells with IC_{50} values determined on adult *S. mansoni*.

The cytotoxic potential of two lead candidates was additionally determined on human cervical carcinoma cells (HeLa) cells and human fetal Lung fibroblast cells (MRC-5) (see Supporting Information).

In Vivo Testing. Compounds revealing an activity of ≤15 μM on adult worms post 72 h drug exposure were tested in vivo. Groups of four infected NMRI mice characterized by a patent schistosome infection (49 days post infection) were treated orally with the test drug using single oral doses of 400 mg of compound per kg body weight. Eight to ten untreated mice served as controls. Fourteen days post-treatment, animals were killed by the CO_2 method, dissected, and worms were sexed and counted.[7] Worm burdens of treated mice were compared to untreated animals and reductions of worm burden calculated.

Compounds displaying high activities against adult *S. mansoni* in vivo were also tested in the juvenile *S. mansoni* mouse model. For that purpose, mice were treated with the test compounds 21 days post infection. Mice were killed and dissected four weeks post treatment. Worm burden reductions were calculated as described above.

Statistics. Parasite viability values of treated and untreated NTS and adult schistosomes obtained from sextuplicate evaluation were averaged (means (± standard deviation)) using Microsoft Excel software. IC_{50} values of test compounds were determined using the CompuSyn software (version 3.0.1, 2007; ComboSyn, Inc.). The Kruskal−Wallis test was applied for in vivo studies, comparing the medians of the worm burden reductions of the treatment and control groups. A difference in median was considered to be significant at a significance level of 5% (StatsDirect statistical software, version 2.7.2.; StatsDirect Ltd., United Kingdom).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^1H and ^{13}C NMR spectra, data of elemental analysis, and physical state of newly introduced tetraoxanes, structure **64** and cytotoxic potential of lead candidates on human cell lines. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

NTS, newly transformed schistosomula; WBR, worm burden reduction; TWBR, total worm burden reduction; FWBR, female worm burden reduction; SI, selectivity index

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Chapter 6

Preclinical studies of Praziquantel Derivatives

6.1 Ferrocenyl Derivatives of the Anthelmintic Praziquantel: Design, Synthesis, and Biological Evaluation

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Ferrocenyl Derivatives of the Anthelmintic Praziquantel: Design, Synthesis, and Biological Evaluation

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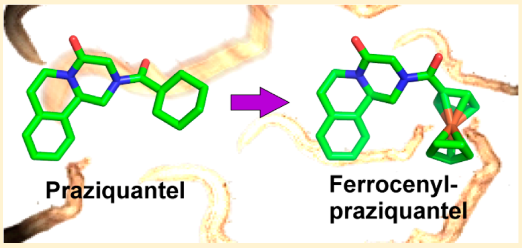
Supporting Information



ABSTRACT: The design, synthesis, and biological evaluation of 18 ferrocenyl derivatives (**4A**–**12A** and **4B**–**12B**) of the most well-known drug against schistosomiasis, namely praziquantel (PZQ), are reported. These compounds, which have been all isolated as racemates, were unambiguously characterized by ^{1}H and ^{13}C NMR spectroscopy, mass spectrometry, and elemental analysis as well as by X-ray crystallography for **4A**, **5A**, and **7A**. Cytotoxicity studies revealed that the complexes were moderately toxic toward a cervical cancer cell line (HeLa) and, importantly, significantly less active toward a noncancerous cell line (MRC-5). The *in vitro* anthelmintic activity of the 18 ferrocenyl PZQ derivatives was tested against adult *Schistosoma mansoni*, and values in the micromolar range (26–68 μM) were determined for the four most active compounds. It was also demonstrated using two compounds of the series as models (**8A** and **8B**) that the complexes were stable when incubated for 24 h at 37 °C in human plasma.

INTRODUCTION

Schistosomiasis is a parasitic disease caused by trematodes of the genus *Schistosoma*. It is a major health problem worldwide, particularly in tropical regions where 280 000 deaths are reported annually.[1,2] In addition, more than 207 million people, mostly in Africa, are infected, and nearly 800 million are at risk of being infected.[3] Currently, praziquantel (PZQ, Figure 1) is widely used, as a racemic mixture, to control this infection in human as well as in animals. PZQ exhibits a broad spectrum anthelmintic activity against all five human *Schistosoma* species. Although no clear evidence exist to date, the voltage-gated Ca^{2+} channels in the membrane are believed to be one of the possible targets for PZQ.[4,5] Despite its success, PZQ suffers from several drawbacks. For example, its metabolic stability is rather low. PZQ is indeed rapidly converted *in vivo* into the less active or inactive PZQ-OH (Figure 1) by hydroxylation of the cyclohexane ring.[6,7] Furthermore, PZQ is inactive against juvenile schistosomes; hence, it is necessary to retreat patients after a few weeks to remove those parasites that have since matured.[5] But potentially more worryingly, as millions of people are regularly treated with this drug, it is likely PZQ resistant parasites emerge in the near future.[4,5] Indeed, reduced susceptibility of *Schistosoma mansoni* to PZQ was already observed.[5,8]

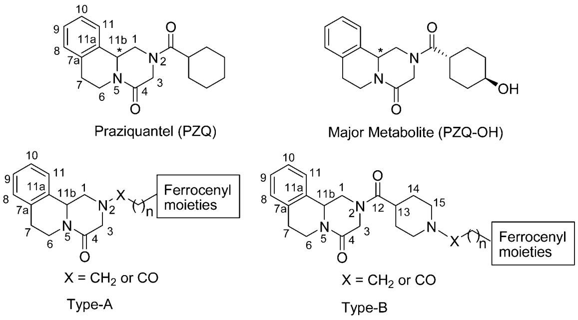


Figure 1. Structures including atom numbering of PZQ and of its major metabolite PZQ-OH as well as of the Fc-PZQ derivatives (type-A and -B) studied in this report.

In order to overcome these drawbacks, chemical modifications on the PZQ structure were undertaken. For example, Todd et al. reported several PZQ derivatives with a modification at the C10 position of the aromatic ring (see Figure 1 for the atom numbering in the PZQ structure).[9] They concluded that the aromatic part is not a suitable position for structural modification. Robert and Meunier et al. disclosed the synthesis and biological evaluation of trioxaquantels which are hybrid molecules made of the 1,2,4-trioxane unit of the antimalarial drug artemisinin and the pyrazinoisoquinoline

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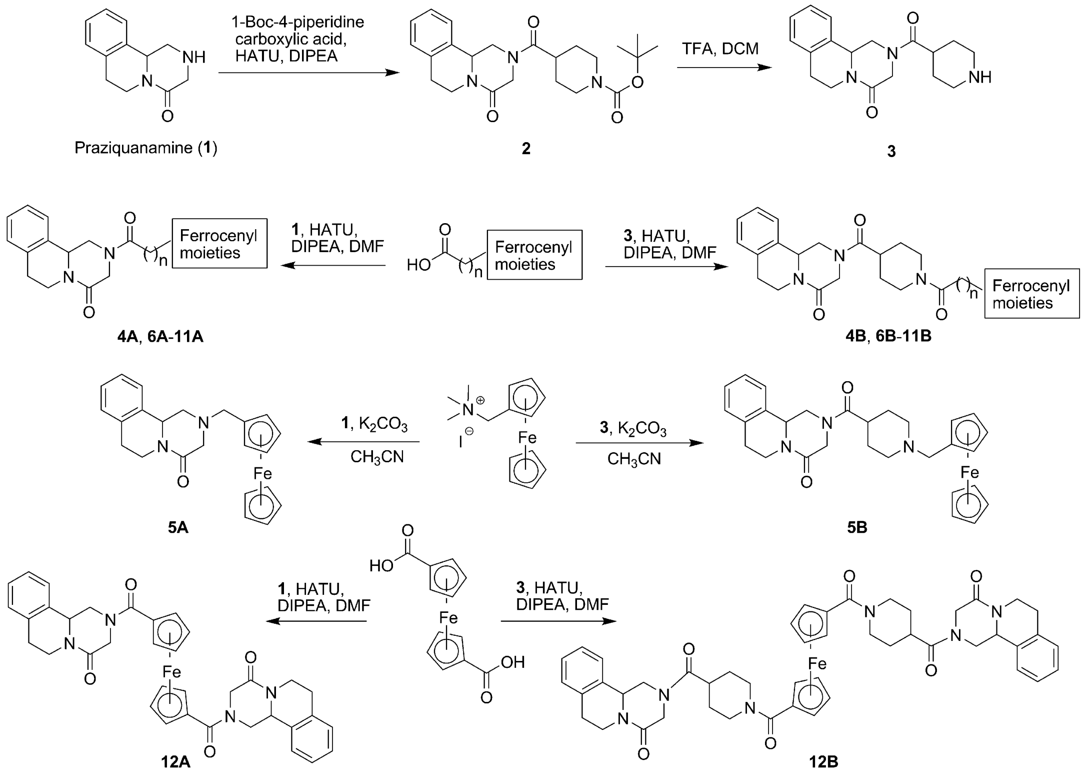
Scheme 1. Synthesis of Fc-PZQ Derivatives



moiety of PZQ.[10] Only one of their compounds was found to have a moderate antiparasitic activity. Recently, Domling et al. reported the synthesis of PZQ derivatives using a Ugi 4-component reaction,[11] and they observed that a few of their analogues were as active as the parent drug PZQ. Of high interest, Vennerstrom and co-workers recently prepared PZQ derivatives with activity against the juvenile stage of *S. mansoni*.[12] Nonetheless, to the best of our knowledge, no lead compound suitable for further preclinical testing was identified in these studies. This fact clearly emphasizes the need for a fundamentally different approach for the discovery of novel PZQ derivatives active against schistosomiasis.

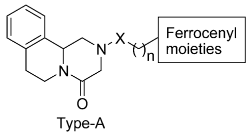
With this in mind, we envisaged to derivatize PZQ with organometallic moieties as previously undertaken with anticancer, antibacterial, and antimalarial drugs.[13−26] This strategy was found to be very successful with ferroquine (FQ), a ferrocenyl analogue of the antimalarial drug chloroquine (CQ), as FQ is active on CQ-resistant *Plasmodium falciparum* strains. Furthermore, intensive chemical biology studies have recently allowed unveiling additional modes of action for FQ compared to the parent drug, which were attributed to the presence of the organometallic unit.[19,27] It is anticipated that these additional modes of action will help prolong the period of resistance development. Of note, Sánchez-Delgado and co-workers reported the preparation of ruthenium half-sandwich analogues of CQ which were found to be highly potent and to overcome the CQ resistance.[28] Organometallic compounds have also been investigated for their activity against other parasites such as the trypanosome *Trypanosoma cruzi* (*T. cruzi*) or *Echinococcus multilocularis* metacestodes.[29−31] For example, a ruthenium half-sandwich complex of the antifungal drug clotrimazole was found to be 58 times more potent than the parent drug in vitro against *T. cruzi*.[29]

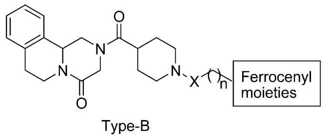
Due to these promising results obtained with other parasitic drugs, we have embarked on a project to evaluate the potential of ferrocenyl derivatives of PZQ against *S. mansoni*. In analogy to what observed for FQ, we postulated that the derivatization of PZQ with a ferrocenyl moiety could potentially prevent future resistance as well as render the compound active against the juvenile stage of the parasite which is refractory to PZQ. In this article, we report our initial results on the synthesis and biological activity of the first organometallic derivatives of PZQ. As shown in Figure 1, our concept was to replace the cyclohexane ring of PZQ with a ferrocenyl unit as it has been reported that the replacement of the cyclohexane ring of PZQ by a benzene ring was not significantly altering the activity.[9,11,32] Moreover, we anticipated that the *in vivo* transformation of PZQ into PZQ-OH could be avoided due to the missing cyclohexane ring in our derivatives. In this study, two different structural classes (type-A and -B, Figure 1) of ferrocenyl-praziquantel (Fc-PZQ) derivatives were investigated. Ferrocenyl derivatives in type-A analogues are directly attached to the praziquanamine (**1**, Scheme 1) residue with different linkers. In contrast, type-B analogues are structurally closer to PZQ as the ferrocenyl moieties are linked to a piperidine unit replacing the cyclohexane ring attached to the praziquanamine via an amide bond (Figure 1).

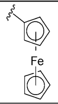
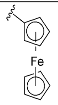
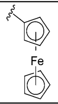
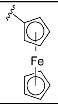
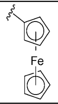
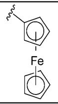
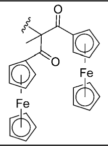
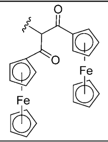
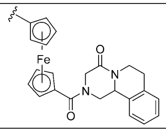
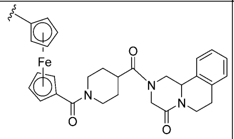
■ RESULTS AND DISCUSSION

Synthesis. The syntheses of the Fc-PZQ analogues are schematically presented in Scheme 1. For more details on the

Table 1. Structures and Yields of Fc-PZQ Derivatives





Fc-PZQ derivatives		Ferrocenyl moieties	X	n	Yield	
Type-A	Type-B				Type-A	Type-B
4A	**4B**		CO	0	62%	75%
5A	**5B**		CH_2	0	58%	89%
6A	**6B**		CO	1	36%	25%
7A	**7B**		CO	2	65%	53%
8A	**8B**		CO	3	73%	65%
9A	**9B**		CO	4	64%	78%
10A	**10B**		CO	2	78%	55%
11A	**11B**		CO	2	63%	49%
12A			CO	0	44%	
	12B		CO	0		56%

structure of the compounds, the readers are referred to Table 1. It should be also noted that all chiral compounds described in this study are racemates. Specifically, praziquanamine (**1**) was prepared following a previously reported literature procedure.[10]Compound **1** was then converted into **3** by treatment with 1-Boc-4-piperidinecarboxylic acid followed by the Boc group deprotection with TFA. All ferrocene-containing carboxylic acid derivatives[33−35] and trimethyl(ferrocenylmethyl)ammonium iodide[36] are either commercially available or were prepared following standard literature procedures. The ferrocene-containing carboxylic acids were then attached to either **1** or **3** via a HATU mediated amide coupling reaction to provide **4A**, **6A−12A** and **4B**, **6B−12B**, respectively. Compounds **5A** and **5B** were prepared by treatment of **1** and **3**, respectively, with trimethyl(ferrocenylmethyl)ammonium iodide and K_2CO_3 in acetonitrile using a synthetic method similar to what employed by Spiccia et al.[37] All new compounds were unambiguously characterized using ^1H and ^{13}C NMR spectroscopy, ESI mass spectrometry, and elemental analysis (see Experimental Section and SI). The presence of rotamers in solution for all Fc-PZQ derivatives except for **4A**, **5A**, and **12A** was ascertained by analysis of their ^1H and ^{13}C NMR spectra. In ESI-mass spectrometry (positive detection mode), most of the compounds were identified as their $[M + Na]^+$ species or, in a few exceptions, as their $[M + H]^+$ or $[M + K]^+$ species. Of note, all ferrocenyl compounds were found to be soluble in aqueous solutions containing 0.5% of DMSO up to 100 μM concentration.

X-ray Crystallography. The molecular structures of **4A**, **5A**, and **7A** were confirmed by the determination of their respective X-ray single crystal structures. All the compounds crystallized as their racemates. ORTEP representations are shown in Figures 2, S1, and S2, respectively. Table S1 (in the SI) contains the relevant crystallographic data and parameters. For the three Fc-PZQ derivatives, the interatomic distances and angles are typical for such compounds.[38] For example, the average distances between the carbon atoms of the Cp rings of ferrocene and iron fluctuate between 2.026 and 2.059 Å for **4A**.

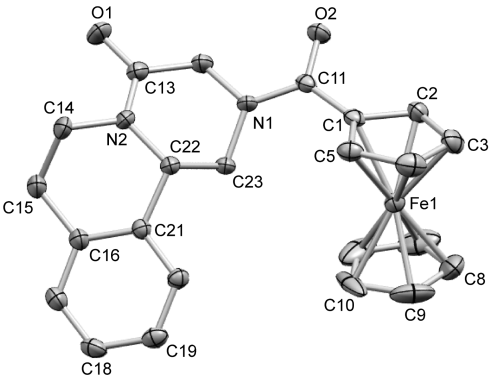


Figure 2. Molecular structure of **4A** (one enantiomer is shown), showing the numbering scheme and the displacement ellipsoids drawn at the 50% probability level. Hydrogen atoms are omitted for clarity.

Biological Evaluation. An ideal antiparasitic agent is supposed to kill selectively the parasites without, or to a significantly less extent, being harmful to the host. We therefore evaluated the toxic behavior of our Fc-PZQ derivatives toward two mammalian cell lines, namely a cervical cancer cell line (HeLa) and a noncancerous cell line (MRC-5). Cisplatin, a platinum based anticancer drug, was used as a reference compound. The resulting IC_{50} values are summarized in Table 2. In general, the ferrocenyl compounds studied in this work were found to be moderately cytotoxic toward HeLa cells. Interestingly, a significant decrease in cytotoxicity was observed for all compounds except **11B** when their activity was analyzed on a nontransformed cell line. This shows an interesting selectivity toward cancer cells. Among all the compounds tested, **9A**, which has the longer CH_2-linker between the praziquanamine and the ferrocenyl moiety, inhibited HeLa cell viability with a half inhibitory concentration (IC_{50}) of 16.9 ± 1.0 μM, thus appearing to be the most cytotoxic compound of this study. The IC_{50} value displayed by **9A** was comparable to that of cisplatin (IC_{50} = 11.5 ± 2.9 μM). Interestingly, **9A** was much more selective toward the cancer cell line studied in this work compared to cisplatin. Similar results were obtained for **9B**. Shortening the length of the linker between the organic and the ferrocenyl moiety resulted in reduced anticancer activity (**9** > **8** > **7** > **6**, Table 2), a behavior that could be attributed to a decrease in lipophilicity. Such a trend was further confirmed with **12A**, which was more cytotoxic than **4A** despite the presence of a similar linker. However, the presence of two praziquanamine moieties attached to the ferrocenyl core in **12A** makes the compound certainly more lipophilic compared to **4A**.

We then tested the Fc-PZQ derivatives (**4A**/**B**−**12A**/**B**) against adult *S. mansoni* to assess their anthelmintic potential. PZQ was used as a reference compound, and the results obtained are listed in Table 2. Fourteen compounds did not show activity at the highest concentration tested (30 μg/mL). Four compounds (**5A**, **7**−**9A**), which displayed activity at 30 μg/mL, were further studied and IC_{50} values of 25.6−68 μM determined. The highest activity was observed with **8A**, which displayed an IC_{50} of 25.6 μM. All compounds were considerably less active than PZQ (0.1 μM).

Table 2. Anthelmintic Activity against adult *S. mansoni* and Cytotoxicity against HeLa and MRC-5 Cells of Fc-PZQ Derivatives

	IC_{50} values against *Schistosoma mansoni* (μM)		IC_{50} values for two different cell lines (μM)			
			HeLa		MRC-5	
compd	A	B	A	B	A	B
4	>72[b]	>70[b]	>100	>100	>100	>100
5	68	>59[b]	57.9 ± 6.4	81.0 ± 0.2	>100	93.9 ± 2.1
6	>35[b]	>56[b]	81.1 ± 2.2	78.0 ± 0.9	>100	>100
7	51.6	>54[b]	50.6 ± 4.8	ND[a]	>100	ND
8	25.6	>53[b]	24.2 ± 3.5	28.9 ± 0.8	62.3 ± 1.3	70.6 ± 2.8
9	48.6	>52[b]	16.9 ± 1.0	18.9 ± 1.5	37.0 ± 1.9	36.1 ± 2.1
10	>42[b]	>37[b]	>100	ND	>100	ND
11	>43[b]	>37[b]	97.0 ± 4.2	>100	>100	42.4 ± 3.2
12	>47[b]	>35[b]	26.9 ± 1.6	ND	64.7 ± 5.4	ND
PZQ	0.1		>100		ND	
cisplatin	ND		11.5 ± 2.9		7.9 ± 1.2	

[a] ND = not determined. [b] Compound did not show antischistosomal activity at the highest concentration tested (30 μg/mL).

Stability in Human Plasma. In order to obtain preliminary insights into the behavior of our Fc-PZQ derivatives under physiological conditions, the stability of the most active compound **8A** and of its B-type analogue (**8B**) in human plasma was assessed. Specifically, **8A** and **8B** were incubated in human plasma for 24 h at 37 °C, and their stability was checked using an LC-MS technique (see Experimental Section for details).[39] The results were compared with that obtained for the parent drug PZQ in the same assay. As shown in Figures S3−S5 in SI, similar to PZQ, no significant changes were observed either in the UV traces or in the ratio of diazepam (internal standard) and **8A** or **8B** (see table S2 in SI) even after 24 h, suggesting that our Fc-PZQ derivatives are stable under physiological conditions.

■ CONCLUSION

There is undoubtedly an urgent need for the discovery of novel drugs against schistosomiasis. Currently, this parasitic disease which affects millions of people worldwide is successfully treated with a single drug, namely PZQ. However, as the at risk population is regularly being treated with PZQ, there are indications that its widespread use could lead to emergence of PZQ resistant parasites in the near future. In this work, we envisaged an alternative method to enlarge the chemical space of potential drug candidates. Hence, we have successfully derivatized PZQ with different ferrocenyl moieties to give 18 new Fc-PZQ derivatives that were unambiguously characterized including by X-ray crystallography for three compounds. It was demonstrated using two compounds of the series as models (**8A** and **8B**) that the complexes were stable when incubated for 24 h at 37 °C in human plasma. Cytotoxic studies on cancerous (HeLa) and noncancerous (MRC-5) cell lines showed that the Fc-PZQs were significantly less active toward the healthy cell line than the cancer cell line studied in this work, except for one compound. An increase in the cytotoxicity against HeLa cells was correlated with an increase in the length of the linker between the organic and the ferrocenyl moieties, and this for both type-A and -B compounds. The Fc-PZQ compounds were found to have an anthelmintic activity in the micromolar range when tested against *S. mansoni in vitro.* Although the activity of the best compound is not sufficient to proceed into further *in vivo* testing, this study opens new avenues in the search for novel drug candidates against schistosomiasis. Further work with different types of organometallic compounds has been initiated in our laboratories, and our results will be published in due course.

■ EXPERIMENTAL SECTION

Materials. All chemicals were of reagent grade quality or better, obtained from commercial suppliers and used without further purification. Solvents were used as received or dried over molecular sieves. All preparations were carried out using standard Schlenk techniques. Praziquanamine (**1**), 3-ferrocenylpropanoic acid, 4-ferrocenylbutyric acid, 5-ferrocenylpentanoic acid, 4,4-diferrocenoylbutanoic acid, 4,4-diferrocenoylpentanoic acid, and trimethyl-(ferrocenylmethyl)ammonium iodide were prepared following standard literature procedures.[10,33−36] All new compounds whose biological activity was evaluated in this work have a purity ≥95% as confirmed by elemental microanalyses.

Instrumentation and Methods. ^{1}H and ^{13}C NMR spectra were recorded in deuterated solvents on 400 (^{1}H: 400 MHz, ^{13}C: 100.6 MHz) or 500 (^{1}H: 500 MHz, ^{13}C: 126 MHz) MHz spectrometers at room temperature. The chemical shifts, δ, are reported in ppm (parts per million). The residual solvent peaks have been used as an internal reference. The abbreviations for the peak multiplicities are as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet), and br (broad). The ^{1}H and ^{13}C signals were assigned with the help of 2D NMR techniques and by comparison with previously reported NMR spectra of other PZQ derivatives.[11,40] ^{1}H and ^{13}C signal assignments follow the atom numbering presented in Figure 1. ESI mass spectra were recorded on a Bruker Esquire 6000 spectrometer. Elemental microanalyses were performed on a LecoCHNS-932 elemental analyzer.

X-ray Crystallography. Crystallographic data were collected at 183(2) K on an Oxford Diffraction Xcalibur system with a Ruby detector using Mo Kα radiation (λ = 0.7107 Å) that was graphite-monochromated. Suitable crystals were covered with oil (Infineum V8512, formerly known as Paratone N), placed on a nylon loop that is mounted in a CrystalCap Magnetic from Hampton Research and immediately transferred to the diffractometer. The program suite CrysAlis$^{\mathrm{Pro}}$ was used for data collection, multiscan absorption correction, and data reduction.[41] The structures were solved with direct methods using SIR97[42] and were refined by full-matrix least-squares methods on F^2 with SHELXL-97.[43] The structure of **7A** contains 2 molecules in the asymmetric unit; it is racemically twinned, and one of the ferrocene rings had to be refined with the help of DELU restraints. The structures were checked for higher symmetry with help of the program Platon.[44] CCDC 892689−892691 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Stability[39] of 8A, 8B, and PZQ in Human Plasma. The human plasma was provided by the Blutspendezentrum, Zurich, Switzerland. Stock solutions in DMSO of 1.6 mM of **8A**, **8B** and PZQ and of 800 μM of diazepam (internal standard, obtained from Sigma-Aldrich) were first prepared. A 12.5 μL portion of the solution containing the compound to be studied and 12.5 μL of the diazepam solution were added to 975 μL of plasma. The resulting solution was shaken gently (ca. 300 rpm) at 37 °C for 24 h. Afterward, 6 mL of a 2/1 (v/v) methyl-tert-butyl ether/CH_2Cl_2 mixture was added to the plasma solution, and the mixture was shaken for 15 min at room temperature and finally centrifuged at 2000 × *g* at 4 °C for 10 min. Finally, the organic layer was separated from the water phase, and the solvent was evaporated with the help of a nitrogen flow. The resulting residue was dissolved in 130 μL of a 8/5 (v/v) CH_3CN/H_2O mixture containing 0.02% TFA and 0.05% HCOOH. A 40 μL portion of this mixture was then injected into the HPLC (Acquity Ultra Performance LC, Waters) that was connected to a mass spectrometer (Bruker Esquire 6000) operated in ESI mode. The reverse phase column used was a Nucleosil 100-5 C18 column (250 mm × 3 mm) with a flow rate of 0.5 mL min^{-1}, and UV-absorption was measured at 220 nm. The runs were performed with a linear gradient of A (acetonitrile (Sigma-Aldrich HPLC-grade)) and B (distilled water containing 0.02% TFA and 0.05% HCOOH): t = 0−3 min, 40% A; t = 6 min, 50% A; t = 16 min, 90% A; t = 20 min, 100% A; t = 23 min, 100% A; t = 25 min, 40% A.

Cell Culture. Human cervical carcinoma cells (HeLa) cells were cultured in DMEM (Gibco) supplemented with 5% fetal calf serum (FCS, Gibco), 100 U/mL penicillin, 100 μg/mL streptomycin at 37 °C and 5% CO_2. The normal human fetal lung fibroblast MRC-5 cell line was maintained in F-10 medium (Gibco) supplemented with 10% FCS (Gibco), penicillin (100 U/mL), and streptomycin (100 μg/mL).

Cytotoxicity Studies. Cytotoxicity studies were performed on two different cell lines, namely HeLa, and MRC-5, by a fluorometric cell viability assay using Resazurin (Promocell GmbH). Briefly, one day before treatment, cells were seeded in triplicates in 96-well plates at a density of 4 × 10^3 cells/well for HeLa and 7 × 10^3 for MRC-5 in 100 μL growth medium. Upon treating cells with increasing concentrations of Fc-PZQ derivatives for 48 h, the medium was removed, and 100 μL complete medium containing Resazurin (0.2 mg/mL final concentration) was added. After 4 h of incubation at 37 °C, fluorescence of the highly red fluorescent product Resorufin was quantified at 590 nm emission with 540 nm excitation wavelength in a SpectraMax M5 microplate reader.

Determination of Antiparasitic Activity against Adult *S. mansoni*. Studies were approved by the local veterinary agency (permit 2070). Female NMRI mice (*n* = 5, obtained from Harlan Laboratories (Horst, The Netherlands)) were subcutaneously infected with ~100 cercarciae following standard procedures.[45] Seven weeks postinfection adult *S. mansoni* were removed from the hepatic portal system and mesenteric veins and cultured in RPMI 1640 culture medium (supplemented with 5% inactivated fetal calf serum (iFCS) and 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA)) at 37 °C in an atmosphere of 5% CO_2 until usage.

For the determination of activity against adult flukes all drugs were initially tested at a concentration of 30 μg/mL, using DMSO stock solutions (conc 10 mg/mL) diluted in supplemented RPMI 1640 medium within 24 flat bottom well plates (BD Falcon, USA) with a final volume of 2 mL per well. Three worms of both sexes were placed into each well. Wells with the highest concentration of DMSO in medium served as controls. PZQ served as positive control, and concentrations of 0.11, 0.33, 1.1, and 3.3 μg/mL were used to determine the IC_{50} value. Phenotypes were monitored after 72 h using the motility scale described by Ramirez et al.[46] and an inverse microscope (Carl Zeiss, Germany, magnification 80×). Compounds presenting antischistosomal activity were characterized further. Therefore, three additional concentrations of selected test drugs were tested (1.1, 3.3, and 10 μg/mL) as described above. Each experiment was performed at least three times. IC_{50} values of active compounds were calculated with CompuSyn software (Version 3.0.1, 2007; ComboSyn, Inc.) as described before.[47]

■ SYNTHESIS

General Procedure for Amide Coupling (GP-1). To a stirred solution of the carboxylic acid in DMF were added HATU and DIPEA successively, and the mixture was allowed to stir for 30 min under a nitrogen atmosphere. The appropriate amine dissolved in DMF is then added and the mixture stirred at room temperature. The reaction mixture was then diluted with EtOAc and washed with 0.5 M HCl, H_2O, and brine. The organic phase was then dried over anhydrous Na_2SO_4, filtered, and concentrated. Compounds were purified either by washing with diethyl ether or by flash column chromatography on silica gel.

Compound 4A. Compound **4A** was synthesized following GP-1: ferrocene carboxylic acid (113 mg, 0.49 mmol), praziquanamine (100 mg, 0.49 mmol), HATU (279 mg, 0.73 mmol), DIPEA (128 mg, 0.99 mmol), DMF (3 mL), and reaction time (20 h). Flash column chromatography (silica gel, hexane/EtOAc 2/1→0/1) gave **4A** as an orange solid (yield: 127 mg, 62%). R_f = 0.68 (silica gel, EtOAc). ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 2.65−2.71 (m, 1H, H7), 2.77−2.82 (m, 1H, H6), 2.88−2.93 (m, 2H, H1 and H7), 4.01 (d, 1H, H3), 4.16 (s, 5H, C_5H_5), 4.30−4.32 (m, 2H, C_5H_4), 4.55 (s, br, 1H, C_5H_4), 4.59 (s, br, 1H, C_5H_4), 4.73−4.76 (m, 1H, H6), 4.84−4.86 (m, 1H, H11b), 4.93 (d, 1H, H3), 5.02 (m, 1H, H1), 7.12−7.28 (m, 4H, C_6H_4). $^{13}C\{^1H\}$ NMR (126 MHz, $CDCl_3$): δ (ppm) 28.7 (C7), 38.8 (C6), 47.2 (C1), 50.6 (C3), 54.7 (C11), 69.3 (C_5H_4), 69.8 (C_5H_4), 70.0 (C_5H_5), 70.3 (C_5H_4), 71.7 (C_5H_4), 76.2 (C_5H_4), 125.4 (C_6H_4), 126.9 (C_6H_4), 127.5 (C_6H_4), 129.4 (C_6H_4), 132.7 (C_6H_4), 134.9 (C_6H_4), 164.8 (C4), 170.2 (N-CO-C_5H_4). ESI-MS (pos. detection mode) *m/z* (%): 437.1 (80) [M + Na]$^+$, 851.1 (100) [2M + Na]$^+$. Anal. Calcd for $C_{23}H_{22}FeN_2O_2$: C 66.68, H 5.35, N 6.76. Found: C 66.92, H 5.17, N 6.66.

Compound 5A. A mixture of trimethyl(ferrocenylmethyl)-ammonium iodide (285 mg, 0.74 mmol), praziquanamine (100 mg, 0.49 mmol), and K_2CO_3 (126 mg, 0.91 mmol) in CH_3CN (20 mL) was refluxed under N_2 atmosphere. After 16 h, the reaction mixture was cooled to room temperature, and K_2CO_3 was removed by filtration. The solvent was removed, and the resulting residue was subjected to flash column chromatography (silica gel, EtOAc/MeOH 15/1→10/1) to give **5A** as a yellow solid (yield: 115 mg, 58%). R_f = 0.40 (silica gel, EtOAc). ^{1}H NMR (401 MHz, $CDCl_3$): δ (ppm) 2.27−2.33 (m, 1H, H1), 2.72 (m, 1H, H7), 2.82−2.96 (m, 3H, H7, H6, H3), 3.51−3.55 (m, 4H, C_5H_4−CH_2, H1, H3), 4.16−4.22 (m, 9H, C_5H_5 and C_5H_4), 4.77−4.83 (m, 2H, H6, H11b), 7.10−7.29 (m, 4H, C_6H_4). $^{13}C\{^1H\}$ NMR (100.6 MHz, $CDCl_3$): δ (ppm) 28.7 (C7), 38.6 (C6), 55.4 (C11b), 55.5 (C1), 56.4 (C3), 57 (C_5H_4-CH_2), 68.5 (C_5H_4), 68.6 (C_5H_5), 70.0 (C_5H_4), 70.3 (C_5H_4), 81.1 (C_5H_4), 124.6 (C_6H_4), 126.5 (C_6H_4), 126.9 (C_6H_4), 129.2 (C_6H_4), 134.4 (C_6H_4), 134.9 (C_6H_4), 166.4 (C4). ESI-MS (pos. detection mode) *m/z* (%): 423.1 (70) [M + Na]$^+$, 439.0 (40) [M + K]$^+$, 823.2 (100) [2M + Na]$^+$. Anal. Calcd for $C_{23}H_{24}FeN_2O$: C 69.01, H 6.04, N 7.00. Found: C 69.21, H 6.11, N 6.95.

Compound 6A. Compound **6A** was synthesized following GP-1: ferrocenyl acetic acid (200 mg, 0.82 mmol), praziquanamine (234 mg, 1.16 mmol), HATU (440 mg, 1.16 mmol), DIPEA (199 mg, 1.54 mmol), 4 mL of DMF, and 7 h reaction time. Flash column chromatography (filter column on alumina, hexane/EtOAc 4/1→0/1) gave a brown solid. The solid was dissolved in 1:1 hexane/EtOAc mixture and kept at −20 °C for one week. The resulting precipitate was collected by filtration and washed with Et_2O to give **6A** (yield: 128 mg, 36%). Note that the compound was found to be unstable on silica and slowly decomposes on alumina. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 2.74−3.10 (m, 4H, H1, H6, 2 × H7), 3.5 (s, 2H, C_5H_4−CH_2−CO), 3.61−3.72 (min) and 4.08−4.50 (maj) (rotamers, m, 11H, H3, C_5H_5 and C_5H_4), 4.61 (min) and 4.82−4.85 (maj) (rotamers, m, 2H, H6, H11b), 5.09 (m, 1H, H1), 7.10−7.29 (m, 4H, C_6H_4). $^{13}C\{^1H\}$ NMR (100.6 MHz, $CDCl_3$): δ (ppm) 28.7 (maj) and 28.8 (min) (rotamers, C7), 35.1 (maj) and 35.5 (min) (rotamers, C_5H_4−CH_2−CO−N), 38.6 (min) and 39.1 (maj) (rotamers, C6), 45.2 (maj) and 46.2 (min) (rotamers, C1), 49.5 (maj) and 49.9 (min) (rotamers, C3), 54.8 (maj) and 55.3 (min) (rotamers, C11b), 67.8 (min) and 68.0 (maj) (rotamers, C_5H_4), 68.1 (maj) and 68.3 (min) (rotamers, C_5H_4), 68.8 (min) and 68.9 (maj) (rotamers, C_5H_5), 69.2 (C_5H_4), 80.7 (maj) and 81.3 (min) (rotamers, C_5H_4), 125.2 (min) and 125.5 (maj) (rotamers, C_6H_4), 126.9 (min) and 127.0 (maj) (rotamers, C_6H_4), 127.5 (maj) and 127.6 (min) (rotamers, C_6H_4), 129.3 (maj) and 129.6 (min) (rotamers, C_6H_4), 132.2 (min) and 132.6 (maj) (rotamers, C_6H_4), 134.7 (maj) and 135.4 (min) (rotamers, C_6H_4), 164.1 (maj) and 165.1 (min) (rotamers, C4), 169.2 (min) and 169.6 (maj) (rotamers, C_5H_4−CH_2−CO−N). ESI-MS (pos. detection mode) *m/z* (%): 451.1 (100) [M + Na]$^+$. Anal. Calcd for $C_{24}H_{24}FeN_2O_2$: C 67.30, H 5.65, N 6.54. Found: C 67.02, H 5.49, N 6.61.

Compound 7A. Compound **7A** was synthesized following GP-1: 3-ferrocenylpropanoic acid (126 mg, 0.49 mmol), praziquanamine (99 mg, 0.49 mmol), HATU (279 mg, 0.74 mmol), DIPEA (126 mg, 0.98 mmol), 4 mL of DMF, and 7 h reaction time. Flash column chromatography (silica gel, hexane/EtOAc 1/2→0/1) gave **7A** as an orange sticky solid (yield: 141 mg, 65%). R_f = 0.19 (silica gel, hexane/EtOAc 1:3). ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 2.49−2.63 (m, 2H, C_5H_4−CH_2−CH_2−CO), 2.70−2.83 (m, 4H, C_5H_4−CH_2−CH_2−CO, H7, H1), 2.87−2.98 (m, 2H, H6, H7), 3.10−3.15 (min) and 3.93−3.97 (maj) (rotamers, m, 1H, H3), 4.09−4.30 (m, 10H, H3, C_5H_4 and C_5H_5), 4.69 (min) and 4.75−4.84 (maj) (rotamers, m, 2H, H6, H11b), 3.90 (min) and 5.12 (maj) (rotamers, m, 1H, H1), 7.05−7.21 (m, 4H, C_6H_4). $^{13}C\{^1H\}$ NMR (126 MHz, $CDCl_3$): δ (ppm) 25.9 (maj) and 26.3 (min) (rotamers, C_5H_4−CH_2−CH_2−CO), 29.2 (maj) and 29.3 (min) (rotamers, C7), 35.4 (maj) and 35.7 (min) (rotamers, C_5H_4−CH_2−CH_2−CO), 39.2 (min) and 39.6 (maj) (rotamers, C6), 45.6 (maj) and 46.7 (min) (rotamers, C1), 49.6 (maj) and 50.3 (min) (rotamers, C3), 55.4 (maj) and 55.8 (min) (rotamers, C11b), 68.2 (C_5H_4), 68.4 (C_5H_4), 68.7 (min) and 68.9 (maj) (rotamers, C_5H_4), 69.1 (C_5H_4), 69.4 (maj) and 69.9 (min) (rotamers, C_5H_5), 88.1 (C_5H_4), 125.9 (min) and 126.1 (maj) (rotamers, C_6H_4), 127.4 (min) and 127.5 (maj) (rotamers, C_6H_4), 128.1 (maj) and 128.2 (min) (rotamers, C_6H_4), 129.8 (maj) and 130.1 (min) (rotamers, C_6H_4), 132.6 (min) and 133.2 (maj) (rotamers, C_6H_4), 135.3 (maj) and 135.9 (min) (rotamers, C_6H_4), 164.6 (maj) and 165.9 (min) (rotamers, C4), 171.3 (min) and 171.7 (maj) (rotamers, CH_2−CH_2−CO−N). ESI-MS (pos. detection mode) *m/z* (%): 442.1 (70) [M]$^+$, 465.1 (100) [M + Na]$^+$, 481.1 (80) [M + K]$^+$. Anal. Calcd for $C_{25}H_{26}FeN_2O_2$: C 67.88, H 5.92, N 6.33. Found: C 68.09, H 5.78, N 6.19.

Compound 8A. Compound **8A** was synthesized following GP-1: 4-ferrocenylbutyric acid (200 mg, 0.73 mmol), praziquanamine (148 mg, 0.73 mmol), HATU (418 mg, 1.1 mmol), DIPEA (188 mg, 1.46 mmol), 5 mL DMF, and 7 h reaction time. Flash column chromatography (silica gel, hexane/EtOAc 1/2→1/3) gave **8A** as an orange sticky solid (yield: 245 mg, 73%). R_f = 0.23 (silica gel, hexane/EtOAc 1/2). ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 1.74−1.81 (m, 2H, $C_5H_4-CH_2-CH_2-CH_2$), 2.19−2.37 (m, 4H, $C_5H_4-CH_2-CH_2-CH_2$ and $C_5H_4-CH_2-CH_2-CH_2$), 2.67−2.91 (m, 4H, H1, H6, 2 × H7), 3.91−4.02 (m, 10H, H3, C_5H_4 and C_5H_4), 4.08 (min) and 4.22 (maj) (rotamers, d, 1H, H3), 4.71 (m, 2H, H6, H11b), 3.77 (min) and 5.05 (maj) (rotamers, m, 1H, H1), 7.07−7.24 (m, 4H, C_6H_4). $^{13}C\{^1H\}$ NMR (126 MHz, $CDCl_3$): δ (ppm) 26.7 (maj) and 27.1 (min) (rotamers, $C_5H_4-CH_2-CH_2-CH_2$), 29.3 (maj) and 29.4 (min) (rotamers, $C_5H_4-CH_2-CH_2-CH_2$), 29.7 (C7), 33.1 (min) and 33.3 (maj) (rotamers, $C_5H_4-CH_2-CH_2-CH_2$), 39.2 (min) and 39.7 (maj) (rotamers, C6), 45.6 (maj) and 46.7 (min) (rotamers, C1), 49.6 (maj) and 50.2 (min) (rotamers, C3), 55.5 (maj) and 55.6 (min) (rotamers, C11b), 67.9 (maj) and 68 (min) (rotamers, C_5H_4), 68.7 (C_5H_4), 68.1 (C_5H_4), 68.2 (C_5H_4), 68.7 (maj) and 68.9 (min) (rotamers, C_5H_5), 88.7 (C_5H_4), 125.9 (min) and 126.1 (maj) (rotamers, C_6H_4), 127.5 (min) and 127.6 (maj) (rotamers, C_6H_4), 128.1 (maj) and 128.2 (min) (rotamers, C_6H_4), 129.9 (maj) and 130.2 (min) (rotamers, C_6H_4), 132.6 (min) and 133.3 (maj) (rotamers, C_6H_4), 135.3 (maj) and 136 (min) (rotamers, C_6H_4), 164.8 (maj) and 165.9 (min) (rotamers, C4), 171.6 (min) and 172 (maj) (rotamers, CH_2-CH_2-CO-N). ESI-MS (pos. detection mode) *m/z* (%): 479.1 (100) [M + Na]$^+$. Anal. Calcd for $C_{26}H_{28}FeN_2O_2$: C 68.43, H 6.18, N 6.14. Found: C 68.81, H 6.21, N 6.03.

Compound 9A. Compound **9A** was synthesized following GP-1: 5-ferrocenylpentanoic acid (200 mg, 0.69 mmol), praziquanamine (141 mg, 0.69 mmol), HATU (398 mg, 1.05 mmol), DIPEA (180 mg, 1.4 mmol), 5 mL DMF and 7 h reaction time. Flash column chromatography (silica gel, hexane/EtOAc 1/1→0/1) gave **9A** as an orange sticky solid (yield: 210 mg, 64%). R_f = 0.7 (silica gel, EtOAc). ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 1.48−1.52 (m, 2H, $C_5H_4-CH_2-CH_2-CH_2-CH_2$), 1.61−1.68 (m, 2H, $C_5H_4-CH_2-CH_2-CH_2-CH_2$), 2.24−2.34 (m, 4H, $C_5H_4-CH_2-CH_2-CH_2-CH_2$ and $C_5H_4-CH_2-CH_2-CH_2-CH_2$), 2.70−2.93 (m, 4H, H1, H6, 2 × H7), 3.97−4.07 (m, 10H, H3, C_5H_4 and C_5H_5), 4.18 (min) and 4.28 (maj) (rotamers, d, 1H, H3), 4.69−4.79 (m, 2H, H6, H11b), 3.91 (min) and 5.07 (maj) (rotamers, m, 1H, H1), 7.07−7.24 (m, 4H, C_6H_4). $^{13}C\{^1H\}$ NMR (126 MHz, $CDCl_3$): δ (ppm) 25.4 ($C_5H_4-CH_2-CH_2-CH_2-CH_2$), 29.3 (maj) and 29.4 (min) (rotamers, $C_5H_4-CH_2-CH_2-CH_2-CH_2$), 30.0 (maj) and 30.1 (min) (rotamers, C7), 31.4 (maj) and 31.5 (min) (rotamers, $C_5H_4-CH_2-CH_2-CH_2-CH_2$), 33.7 ($C_5H_4-CH_2-CH_2-CH_2-CH_2$), 39.2 (min) and 39.7 (maj) (rotamers, C6), 45.6 (maj) and 46.7 (min) (rotamers, C1), 49.5 (maj) and 50.1 (min) (rotamers, C3), 55.5 (maj) and 55.9 (min) (rotamers, C11b), 67.8 (maj) and 67.9 (min) (rotamers, C_5H_4), 68.7 (maj) and 68.8 (min) (rotamers, C_5H_4), 69.1 (maj) and 69.2 (min) (rotamers, C_5H_5), 89.4 (maj) and 89.5 (min) (rotamers, C_5H_4), 125.9 (min) and 126.1 (maj) (rotamers, C_6H_4), 127.5 (min) and 127.6 (maj) (rotamers, C_6H_4), 128.1 (maj) and 128.3 (min) (rotamers, C_6H_4), 129.9 (maj) and 130.2 (min) (rotamers, C_6H_4), 132.6 (min) and 133.3 (maj) (rotamers, C_6H_4), 135.3 (maj) and 136 (min) (rotamers, C_6H_4), 164.8 (maj) and 166.1 (min) (rotamers, C4), 171.7 (min) and 172.2 (maj) (rotamers, CH_2-CH_2-CO-N). ESI-MS (pos. detection mode) *m/z* (%): 493.1 (100) [M + Na]$^+$. Anal. Calcd for $C_{27}H_{30}FeN_2O_2$: C 68.94, H 6.43, N 5.96. Found: C 68.81, H 6.40, N 5.91.

Compound 10A. Compound **10A** was synthesized following GP-1: 4,4-diferrocenoylpentanoic acid (85 mg, 0.16 mmol), praziquanamine (33 mg, 0.16 mmol), HATU (92 mg, 0.24 mmol), DIPEA (42 mg, 0.32 mmol), 4 mL of DMF, and 7 h reaction time. Flash column chromatography (silica gel, hexane/EtOAc 1/2→1/3) gave **10A** as an orange solid (yield: 98 mg, 78%). R_f = 0.33 (silica gel, EtOAc/hexane 3/1). ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 1.59 (maj) and 1.63 (min) (rotamers, s, 3H, CH_3), 2.23−2.42 (m, 4H, $N-CO-CH_2-CH_2-C$ and $N-CO-CH_2\text{-}CH_2-C$), 2.65−2.89 (m, 4H, H1, H6, 2 × H7), 3.10 (min) and 3.91 (maj) (rotamers, d, 1H, H3), 3.97 (min) and 3.99 (maj) (rotamers, s, 10H, C_5H_5), 4.23−4.32 (m, 5H, H3 and C_5H_4), 4.56 (m, 2H, C_5H_4), 4.66−4.75 (m, 4H, C_5H_4, H6 and H11b), 3.79 (min) and 5.03 (maj) (rotamers, m, 1H, H1), 7.06−7.23 (m, 4H, C_6H_4). $^{13}C\{^1H\}$ NMR (126 MHz, $CDCl_3$): δ (ppm) 22.2 (maj) and 22.3 (min) (rotamers, CH_3), 29.2 (H7), 29.3 (maj) and 29.4 (min) (rotamers, $N-CO-CH_2-CH_2-C$), 33.3 (maj) and 33.8 (min) (rotamers, $N-CO-CH_2-CH_2-C$), 39.3 (min) and 39.6 (maj) (rotamers, C6), 45.7 (maj) and 46.8 (min) (rotamers, C1), 49.5 (maj) and 50.1 (min) (rotamers, C3), 55.5 (maj) and 56.1 (min) (rotamers, C11b), 64.7 (maj) and 64.9 (min) (rotamers, $C(CH_3)$), 70.7 (maj) and 70.8 (min) (rotamers, C_5H_5), 71.4, 71.5 (maj) and 71.6 (min) (rotamers, C_5H_4), 72.2 (C_5H_4), 72.3 (C_5H_4), 72.4 (C_5H_4), 72.5 (maj) and 72.6 (min) (rotamers, C_5H_4), 79.1 (C_5H_4), 125.3 (maj) and 125.4 (min) (rotamers, C_6H_4), 127.5 (C_6H_4), 128.1 (maj) and 128.3 (min) (rotamers, C_6H_4), 129.8 (maj) and 129.9 (min) (rotamers, C_6H_4), 132.6 (min) and 133.3 (maj) (rotamers, C_6H_4), 135.3 (maj) and 136 (min) (rotamers, C_6H_4), 164.6 (maj) and 165.8 (min) (rotamers, C4), 171.5 (min) and 171.9 (maj) (rotamers, $N-CO-CH_2-CH_2-C$), 200.5 ($C-CO-C_5H_4$). ESI-MS (pos. detection mode) *m/z* (%): 733.2 (100) [M + Na]$^+$. Anal. Calcd for $C_{39}H_{38}Fe_2N_2O_4$: C 65.94, H 5.39, N 3.94. Found: C 66.08, H 5.31, N 3.89.

Compound 11A. Compound **11A** was synthesized following GP-1: 4,4-diferrocenoylbutanoic acid (150 mg, 0.29 mmol), praziquanamine (59 mg, 0.29 mmol), HATU (167 mg, 0.44 mmol), DIPEA (76 mg, 0.59 mmol), 4 mL of DMF, and 7 h reaction time. Flash column chromatography (silica gel, hexane/EtOAc 1/2→1/4) gave **11A** as an orange solid (yield: 128 mg, 63%). R_f = 0.65 (silica gel, EtOAc). ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 2.33−2.52 (m, 4H, $N-CO-CH_2-CH_2$ and $N-CO-CH_2\text{-}CH_2$), 2.64−2.87 (m, 4H, H1, H6, 2 × H7), 3.18 (min) and 3.91 (maj) (rotamers, d, 1H, H3), 4.02−4.05 (m, 10H, C_5H_5), 4.24 (m, 1H, H3), 4.45 (m, 4H, C_5H_4), 4.50 (m, 1H, $CH(CO)_2$), 4.65−4.73 (m, 2H, H6, H11b), 4.84−4.87 (m, 4H, C_5H_4), 3.80 (min) and 5.08 (maj) (rotamers, m, 1H, H1), 7.06−7.23 (m, 4H, C_6H_4). $^{13}C\{^1H\}$ NMR (126 MHz, $CDCl_3$, mixture of tautomers): δ (ppm) 25.5 (maj) and 26.4 (min) (tautomers, $N-CO-CH_2-CH_2$), 29.2 (maj) and 29.3 (min) (tautomers, C7), 31.3 ($N-CO-CH_2-CH_2$), 39.3 (min) and 39.6 (maj) (tautomers, C6), 45.7 (maj) and 46.8 (min) (tautomers, C1), 49.3 (maj) and 50.1 (min) (tautomers, C3), 55.4 (maj) and 56.1 (min) (tautomers, C11b), 61.3 (min) and 61.5 (maj) (tautomers, $CH(CO)_2$), 70.5 (min) and 70.6 (maj) (tautomers, C_5H_4), 70.7 (maj) and 70.8, 70.9 (min) (tautomers, C_5H_5), 73.2, 73.3, 73.4, 73.5 (rotamers of tautomers, C_5H_4) 79.4 (maj) and 79.5 (min) (tautomers, C_5H_4), 126.1 (C_6H_4), 127.5 (maj) and 127.6 (min) (tautomers, C_6H_4), 128.1 (maj) and 128.2 (min) (tautomers, C_6H_4), 129.8 (maj) and 130.6 (min) (tautomers, C_6H_4), 132.6 (min) and 133.3 (maj) (tautomers, C_6H_4), 135.3 (maj) and 135.8 (min) (tautomers, C_6H_4), 164.6 (maj) and 165.2 (min) (tautomers, C4), 170.9 (min) and 171.2 (maj) (tautomers, $N-CO-CH_2-CH_2$), 200.4 (maj) and 200.5 (min) (tautomers, $CH(CO)_2$). ESI-MS (pos. detection mode) *m/z* (%): 719.2 (100) [M + Na]$^+$, 735.2 (60) [M + K]$^+$. Anal. Calcd for $C_{38}H_{36}Fe_2N_2O_4$: C 65.54, H 5.21, N 4.02. Found: C 65.71, H 5.30, N 3.94.

Compound 12A. Compound **12A** was synthesized following GP-1: 1,1′-ferrocenedicarboxylic acid (200 mg, 0.73 mmol), praziquanamine (442 mg, 2.19 mmol), HATU (832 mg, 2.19 mmol), DIPEA (282 mg, 2.19 mmol), 10 mL of DMF, and 20 h reaction time. Flash column chromatography (silica gel, EtOAc/MeOH 15/1) gave **12A** as an orange solid (yield: 206 mg, 44%). R_f = 0.13 (silica gel, EtOAc). ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 2.64−2.98 (m, 8H, H1, H6, 2 × H7), 4.01 (m, 2H, H3), 4.34 (m, 4H, C_5H_4), 4.58 (m, 2H, C_5H_4), 4.66 (m, 6H, C_5H_4, H3 and H6), 4.84 (m, 2H, H11b), 4.92 (m, 2H, H1), 7.06−7.12 (m, 8H, C_6H_4). $^{13}C\{^1H\}$ NMR (126 MHz, $CDCl_3$): δ (ppm) 29.3 (C7), 39.5 (C6), 47.3 (C1), 51.4(C3), 55.2 (C11b), 71.6 (C_5H_4), 71.8 (C_5H_4), 72.2 (C_5H_4), 72.5 (C_5H_4), 72.6 (C_5H_4), 74.1 (C_5H_4), 74.2 (C_5H_4), 79.4 (C_5H_4), 125.9 (C_6H_4), 127.5 (C_6H_4), 128.1 (C_6H_4), 129.9 (C_6H_4), 133.2 (C_6H_4), 135.3 (C_6H_4), 165.2 (C4), 169.7 ($N-CO-C_5H_4$). ESI-MS (pos. detection mode) *m/z* (%): 665.3 (80) [M + Na]$^+$, 1307.4 (70) [2M + Na]$^+$. Anal. Calcd for $C_{36}H_{34}FeN_4O_4$: C 67.29, H 5.33, N 8.72. Found: C 67.08, H 5.18, N 8.54.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis and characterization data of compounds **2**, **3**, and **4B**-**12B**; ^{1}H and ^{13}C NMR spectra; molecular structures of **5A** and **7A**; crystallographic data and structure refinement for **4A**, **5A**, and **7A**; UV traces of the LC-MS analysis of PZQ; **8A** and **8B** in human plasma at *t* = 0 min and 24 h; ratios of peak areas of **8A**/diazepam, **8B**/diazepam, and PZQ/diazepam in human plasma at *t* = 0 min and *t* = 24 h; CIF files for compounds **4A**, **5A**, and **7A**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

Fc, ferrocenyl; PZQ, praziquantel; Fc-PZQ, ferrocenyl praziquantel; HATU, 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; ESI-MS, electrospray ionization mass spectrometry; DIPEA, diisopropylethylamine; SAR, structure activity relationship

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6.2 [(h^6-Praziquantel)Cr(CO)$_3$] Derivatives with Remarkable In Vitro Anti-schistosomal Activity

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[(η^6-Praziquantel)Cr(CO)$_3$] Derivatives with Remarkable In Vitro Anti-schistosomal Activity

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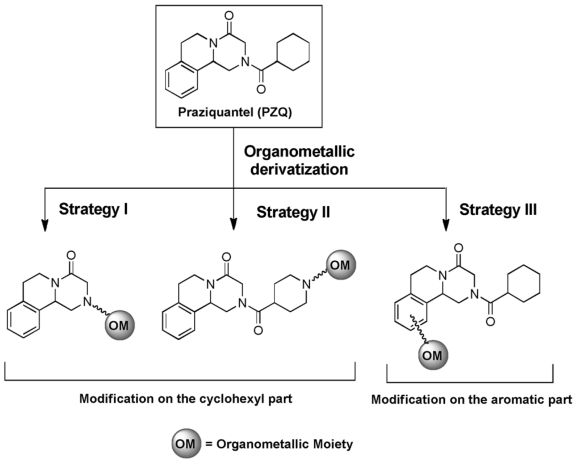
COMMUNICATION

Schistosomiasis is a major human health problem, particularly in rural, tropical regions of developing countries. Every year, 280000 deaths are reported, mostly in sub-Saharan Africa, and more than 207 million people are infected.[1] To further emphasize the socioeconomic significance of this disease, experts consider schistosomiasis as one of the most devastating parasitic diseases after malaria in tropical countries.[2] Currently, a racemic mixture of praziquantel (PZQ, Scheme 1) is the frontline drug for treatment and control of schistosomiasis. PZQ, which is believed to target the voltage-gated Ca^{2+} channels in the membrane of the parasite, exhibits broad-spectrum anthelmintic activity against the main species of *Schistosoma*.[3] However, reduced susceptibility of *Schistosoma mansoni* (*S. mansoni*) to PZQ has been reported recently.[4] This worrying evidence associated to the known drawbacks of PZQ, that is, low metabolic stability in vivo[5] and lack of activity against the juvenile stage of *Schistosoma*,[3b] emphasizes the need for the rapid discovery of alternative drugs to treat schistosomiasis. With this perspective, and concurrently with the investigations of the purely organic modifications of the PZQ structure undertaken by others,[6] our groups have recently initiated a program to derivatize PZQ with organometallic moieties.[7] This strategy was shown to be very successful in the development of anticancer, antibacterial, and antimalarial compounds,[8] with the ferrocenyl analogue of the antimalarial drug chloroquine (CQ), namely ferroquine, being the best example. Ferroquine, which is licensed by the pharmaceutical company Sanofi, was found to be active against CQ-resistant strains of *Plasmodium falciparum* by different metal-specific modes of action.[9] Furthermore, ruthenium half-sandwich complexes were also tested for their anti-parasitic activity against *Trypanosoma cruzi* (which is responsible for Chagas disease), with promising results in vitro.[10]



Scheme 1. Structures of praziquantel (PZQ) and derivatives of PZQ with organometallic moieties that were designed using three different strategies.

In an initial study, we envisaged either replacing or modifying the cyclohexyl moiety of PZQ with different ferrocenyl derivatives (Scheme 1, Strategies I and II). Using this strategy, of eighteen ferrocenyl-PZQ derivatives made, only two showed moderate anthelmintic activity against *S. mansoni* in vitro.[7] Herein, we present an alternative strategy that employs the organometallic derivatization on the aromatic part of PZQ (Scheme 1, strategy III). For this purpose, we selected the $\{Cr(CO)_3\}$ moiety as the fragment to be attached to PZQ for following reasons. First, the preparation of $[(\eta^6\text{-arene})Cr(CO)_3]$ derivatives is usually straightforward and the resulting compounds are, in most cases, air- and water-stable. Second, we anticipated that the attachment of this organometallic moiety may improve the physicochemical properties of the parent drug (enhancement of lipophilicity, increase of metabolic stability and/or alteration of structural and electronic properties of the aromatic part of PZQ). Worthy of note, different bioorganometallic compounds containing the $\{Cr(CO)_3\}$ entity were shown earlier to be useful in field of receptorology and more recently in diverse areas of medicinal chemistry including as antimicrobial, anti-inflammatory, or antimalarial agents.[11] These findings contrast to the common believe that all chromium complexes are toxic. The toxicity of chromium complexes mainly depends on the oxidation state of the metal center, nature of the ligand, solubility, and the dosage provided. Importantly, the Cr^{III} salts that can be potentially formed by light- or oxygen-induced oxidative decomposition of $[(\eta^6\text{-arene})Cr(CO)_3]$ derivatives in solution are relatively less or non-toxic compared to the highly toxic Cr^{VI} species.[11g,12] It is also important to note that other "feared" metals, such as arsenic, antimony, gold, silver, and bismuth, have been used or are still currently being used in diverse forms in medicines.[11g,13] One of the best examples is the arsenic-containing organometallic compound Salvarsan, which was used against syphilis until the 1940s, before penicillin reached the market.[8b,13a]

In this study, the antischistosomal effects of two $[(\eta^6\text{-PZQ})Cr(CO)_3]$ (Cr-PZQ) derivatives (**1** and **2**; Scheme 2)

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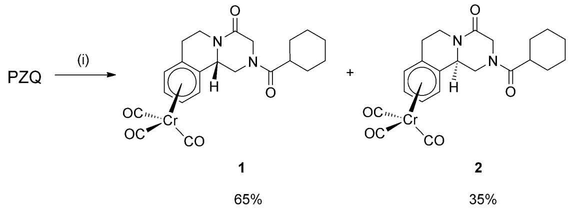

Scheme 2. Synthesis of Cr-PZQ derivatives **1** and **2**. Note that all of the compounds are racemic. Reaction conditions: i) $[Cr(CO)_6]$, Bu_2O, THF, 140 °C.

were investigated. These compounds were prepared in a one-step procedure using commercially available PZQ (Scheme 2). PZQ was heated with $[Cr(CO)_6]$ at 140 °C to yield the diastereomeric mixture of **1** and **2** in 77 % combined yield (note that both **1** and **2** are racemates); **1** and **2** were found to be easily separable by silica-gel flash column chromatography. After purification, the ratio between **1** and **2** was determined to be 65 and 35 %, respectively. As expected, for both diastereomers, an upfield shift in the aromatic proton signals compared with that of PZQ was observed in their ^1H NMR spectrum (see the Supporting Information). ESI-MS spectra as well as the elemental analysis confirmed unambiguously the presence of the expected compounds. The designation was facilitated by the determination of the X-ray single-crystal structure of **2**. Compound **2** crystallized as a racemic mixture, and the ORTEP representation of one of the enantiomers is presented in Figure 1. The chromium tricarbonyl moiety is *trans* to the proton H18 linked to the chiral carbon C18. Furthermore, the structure of **2** does not contain any unusual structural features.[11a]

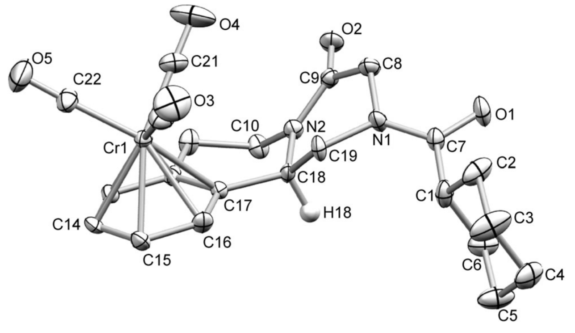

Figure 1. X-ray single-crystal structure of **2** (only one enantiomer is shown). Ellipsoids are set at 50 % probability; hydrogen atoms (except H18) are omitted for clarity.

Having the Cr-PZQ derivatives in hand, we first determined the distribution coefficients of **1** and **2** using the “shake-flask” method. The presence of chromium in **1** and **2** allows their detection in the aqueous phase by atomic absorption spectroscopy (see experimental section in the Supporting Information for further details). As expected, owing to the presence of a $\{Cr(CO)_3\}$ moiety, the lipophilicity of both **1** ($\log D_{7.4}=3.49$) and **2** ($\log D_{7.4}=3.59$) are significantly higher than that of PZQ ($\log D_{7.4}=2.66$).[14] The membrane permeability is therefore expected to be higher for the Cr-PZQ derivatives compared to that of PZQ. The in vitro anthelmintic potential of **1** and **2** was then tested against adult *S. mansoni*, using PZQ as a control. Table 1 shows antischistosomal effects in the nanomolar range for both **1** and **2**. These antischistosomal activities were comparable to that of the parent drug PZQ ($IC_{50}=0.1$ μM). The toxicity of **1** and **2** on mammalian cells was then assessed on the cervical cancer (HeLa) and non-cancerous (MRC-5) cell lines (Table 1). Both compounds were moderately cytotoxic to HeLa cells but not toxic to MRC-5 cells. The high ratio (>270) calculated as the highest activity of **1** and **2** on HeLa cells (68.5 μM) divided by the anthelmintic activity of **1** and **2** (0.25 μM) is a good indication of the selectivity of **1** and **2** to schistosomes.

Table 1. In vitro activity of Cr-PZQ derivatives against adult *S. mansoni* and cytotoxicity against HeLa and MRC-5 cells.

Compound	Anthelmintic activity against *S. mansoni* (μM)	r*[a]	IC_{50} values (μM)	
			HeLa	MRC-5
1	0.25	0.96	68.5 ± 3.0	>100
2	0.27	0.97	81.4 ± 1.5	>100
PZQ	0.10	0.99	>100	>100

[a] r* is the goodness of fit, which is required to be ≥0.85.[15]

To exclude that the promising antischistosomal activities of **1** and **2** achieved in vitro were not due to the release of praziquantel, the stability of the Cr-PZQ derivatives in water was assessed. For this purpose, **1** and **2** were dissolved in a $[D_6]DMSO/D_2O$ mixture and kept in the dark. ^1H NMR spectra were then studied at different time intervals, and the presence of PZQ as a decomposition product of **1** and **2** was assessed. Very little or no PZQ was detected after two days (Supporting Information, Figures S1, S2); **1** was relatively stable and only about 8 % of PZQ was released after 27 days. By comparison, **2** was shown to decompose at a higher rate (ca. 22 % release in the same time). Furthermore, to further confirm the results obtained with these NMR studies, the stabilities of the Cr-PZQ derivatives in human plasma were also evaluated. Consistent with findings for the parent drug PZQ,[7] no significant change was observed either for the UV traces or the ratio between diazepam (internal standard) and **1** or **2** (Figure 2; Supporting Information, Figure S3, S4) up to 24 h, suggesting that the Cr-PZQ derivatives are relatively stable in biological medium such as serum. These results infer that chromium tricarbonyl complexes are stable and the in vitro activity was exhibited by the Cr-PZQ derivatives but not by the released PZQ.

In conclusion, we have demonstrated that two easy-to-prepare chromium tricarbonyl PZQ derivatives achieve an impressive antischistosomal effect (nanomolar range) on adult *S. mansoni* in vitro. Importantly, these compounds

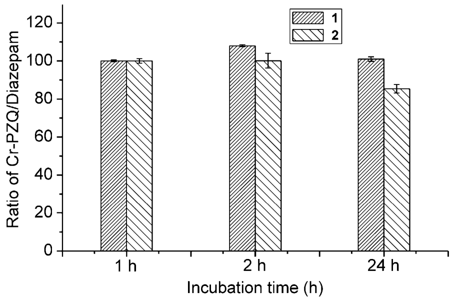

Figure 2. Ratio [%] of Cr-PZQ derivatives **1** and **2** to diazepam (internal standard) at different incubation times in human plasma.

were shown to be safe when tested using two distinct cell lines and had remarkable selectivity for the adult stage of *S. mansoni* that lives in the portal and mesenteric vein system of the human host.[16] Moreover, the stability of both compounds in human serum was confirmed by LC-MS measurements. No significant decomposition was observed when the compounds were incubated in human plasma at 37 °C for 24 h. These findings therefore strongly contrast with the current belief that organometallic compounds, and more importantly, chromium tricarbonyl complexes, are unstable and/or cytotoxic. The results presented herein is another contribution to the booming field of research of medicinal organometallic chemistry[17] and pave the way for a systematic investigation of the structure-activity relationship of organometallic derivatives of PZQ as well as in vivo testing of such compounds.

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Keywords: arene complexes · chromium · medicinal chemistry · praziquantel · schistosomiasis

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Chapter 7

General Discussion and Conclusion

1 General Discussion

Rationale, Objectives and Key findings

The major aim pursued in this thesis was to support antischistosomal drug discovery by improving the screening process itself, by further investigating diverse known lead compounds and by identifying entirely new chemical scaffolds for the empty antischistosomal pipeline (Figure 1).

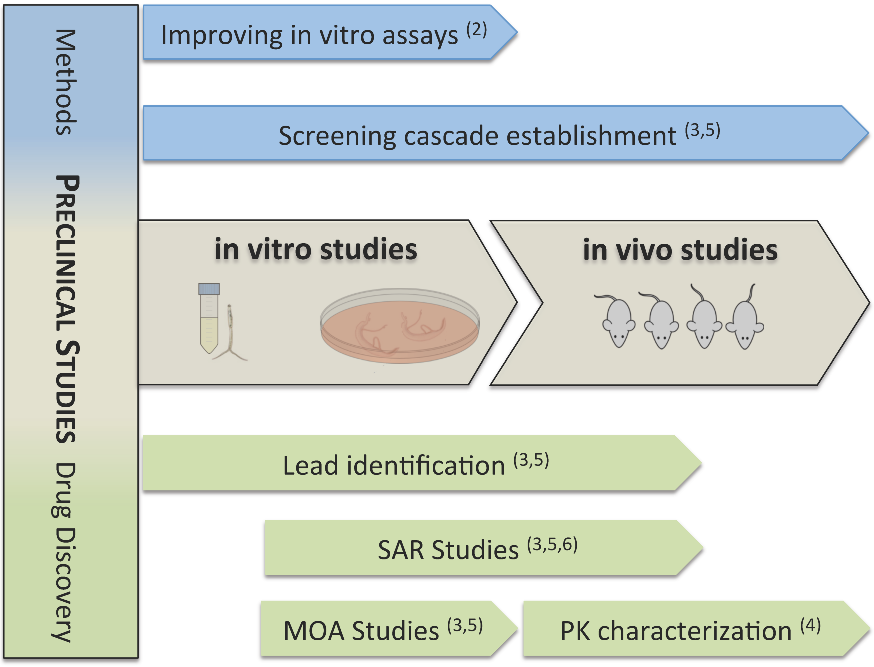

Figure 1: The different aspects of pre-clinical antischistosomal drug discovery presented here were tackled in my PhD thesis. This figure highlights the interrelation of the executed objectives; numbers in brackets indicate the chapter in which each aspect is described. (SAR: Structure Activity Relationship; MOA: Mode of Action; PK: Pharmacokinetic)

There is no doubt that especially drug development for schistosomiasis has been neglected in the last decades (Caffrey and Secor, 2011; Keiser and Utzinger, 2007a, 2012). The availability of praziquantel (PZQ) since the 1970’s as a safe, affordable and single oral dose drug, which has not presented any decrease in clinical drug efficacy yet, might have masked the interest in antischistosomal drug discovery (Caffrey and Secor, 2011). Various challenges for research on schistosomes such as the parasite itself, its complicated lab cultivation and the lack of validated HTS screening systems contribute to the deceleration of innovations (Keiser, 2010; Nwaka et al., 2009). Hence, the current R&D pipeline for schistosomiasis remains weak and potential backup drugs should be identified whilst PZQ is still effective (Keiser and Utzinger, 2012).

Initially we undertook a systematic literature review to identify the bigger picture of needs and shortcomings for the use of antiparasitic drugs in at-risk populations, infants and school-aged children (Appendix 8.1). As expected, the minority of studies (3%) dealt with drugs for schistosomiasis. Merely one PK study on anthelmintics was identified, underlining the total negligence of PK studies with antiparasitic drugs in infants and adolescents. Therefore it should be of high priority and future research interest to assess the impact of parasitic infections on the disposition kinetics of applied drugs (Botros et al., 2011). A major requirement for a fruitful drug pipeline is the improvement of available tools for antischistosomal drug discovery. To contribute to this requisite, the potential use of *S. haematobium* schistosomula for drug screenings was investigated. A schistosomula assay based on microscopic readout was established (Chapter 2). However it was not possible to use resazurin as a robust colorimetric readout. To note, in general we found that colorimetric markers are not ideal for the readout of schistosomula drug assays.

For the elucidation of new chemical scaffolds and lead candidates with antischistosomal activity, we focused on “low hanging fruits”: repositioning of compounds (Chapter 3, marketed antimalarials) or compounds belonging to chemical classes with known antischistosomal activity (Chapter 5, synthetic peroxides; Chapter 6, PZQ derivatives) (Kraus, 2008; Tobinick, 2009).

Only recently mefloquine (MFQ) was identified as a potent new antischistosomal lead candidate. Thereafter preclinical studies investigated phenotypic changes of the parasites after MFQ exposure, activities of MFQ-enantiomers, combination therapy with PZQ and provided insights into the mechanism of action (MOA) on schistosomula (Keiser et al., 2009; Keiser et al., 2011; Manneck et al., 2010; Manneck et al., 2012). Finally its efficacy was proven in clinical trials performed in *S. haematobium* patients (Keiser et al., 2010). Therefore MFQ presented an optimal lead candidate for further preclinical investigations such as SAR and MOA studies. Thereupon nine MFQ-related arylmethanols belonging to the quinolinemethanols, pyridinemethanols and phenanthrenmethanols were selected (Chapter 3.1). The class of 4-quinolinemethanols was found to be the most active class against schistosomes in vitro and the 4-pyridinemethanols were identified as a novel class with antischistosomal activity. Interestingly the two lead 4-quinolinemethanols, MFQ and WR7930, showed tenfold decreased IC_{50} values when incubated in the presence of hemoglobin. Possible interference with the hemozoin formation seems therefore very probable as revealed for quinine and quinidine recently, being likewise 4-aminoquinolines (Correa Soares et al., 2009). The 4-pyridinemethanols did not alter their in vitro activity in the presence of hemoglobin, heme or red blood cells.

Among the arylmethanols tested, two 4-quinolinemethanols (WR7930, MFQ) and one 4-pyridinemethanol (enpiroline (EP)), showed excellent in vivo activity against *S. mansoni* and *S. haematobium* in mice. However WR7930 has been described as mediator of phototoxicity. Hence EP and MFQ, were defined as lead candidates, presenting excellent oral in vivo efficacy against major *Schistosoma* species and adequate safety data are available (Keiser and Utzinger, 2007a). As concluded in the performed literature review described above, only very little is known about the impact of infection on PK-profiles of applied drugs. Therefore PK-profiles of EP and MFQ were determined in infected and non-infected *S. mansoni* mice (Chapter 4). The infection triggered an increase in half-life and AUC, resulting in significantly slower clearance of both drugs. Rather late onsets of action were observed for both drugs (>3 days), which is in line with the extensive long half-lives observed.

The confirmation that antimalarials might be an interesting starting point for antischistosomal drug discovery paved the way for the collaboration with MMV. The open access malaria box, containing 200 drug-like compounds for oral application and 200 probe-like compounds with proven activity against *P. falciparum*, was investigated in vitro and in vivo, and lead candidates were characterized further. This project identified two entirely new chemical scaffolds- the N'-N'-diarylureas and 2,3-dianilinoquinoxalines (Chapter 3.2). Furthermore new insights into in vitro-in vivo and PK-PD correlations could be drawn from the project, discussed in detail below.

The antischistosomal activity of the artemisinins (especially on the juvenile parasite stages) with their distinct peroxidic scaffold (1,2,4 trioxane) has been known for quite a long time (Keiser and Utzinger, 2007b; Utzinger et al., 2007).

The discovery of fully synthetic 1,2,4 trioxanes as antimalarial lead candidates prompted investigation of the ozonides on schistosomes (Vennerstrom et al., 2004; Xiao et al., 2007). Previous studies elucidated OZ288 as a lead candidate (Xiao et al., 2007). We investigated eleven OZ288 analogs in SAR studies. OZ418 displayed excellent in vivo activity on juvenile and adult *S. mansoni* and *S. haematobium* infections in mice (Chapter 5.1).

Another class of chemically stable cyclic peroxides, namely the 3-alkoxy-1, 2-dioxolanes, has previously demonstrated significant in vitro antimalarial activity (Schiaffo et al., 2011). Hence we investigated a set of this chemical class for antischistosomal activity. These compounds revealed interesting in vitro activity on adult schistosomes and NTS, but lacked activity on juvenile and adult *S. mansoni* infections in mice. Non-peroxidic analogues were inactive in vitro, underlining the necessity of the peroxide functional group as an essential pharmacophor, as described for trioxolanes (Xiao et al., 2007). However, supplementation of the medium with iron sources did not alter the in vitro activity, supporting a non-iron dependent mode of activation (Chapter 5.2).

Despite the evaluation of several peroxidic compound classes for their effect on schistosomes in recent years, only little is known on the relationship between the peroxidic structures and antischistosomal activity. Therefore, three new peroxide classes, namely bridged 1,2,4,5-tetraoxanes, alphaperoxides and tricyclic monoperoxides, were investigated in vitro and in vivo against *S. mansoni*. High activity was observed on the schistosomular stage but lower susceptibility was documented on adult worms. As noted above, studies with hemin and heme supplemented medium indicated that antischistosomal activation of peroxides might not be triggered by iron porphyrins. Two compounds (tricyclic monoperoxide; bridged 1,2,4,5-tetraoxane) revealed high worm burden reductions in the chronic but only moderate activity in the juvenile *S. mansoni* mouse model. The low activity of the 3-alkoxy-1, 2-dioxolanes, tetraoxanes and tricyclic monoperoxides on the juvenile stages is contrary to the activity profile of OZs and the artemisinins (Utzinger et al., 2007; Xiao et al., 2007). As noted before, PZQ shows some major limitations in terms of efficacy, most importantly its lacking activity on the juvenile stage. Additionally the oral drug formulation has drawbacks: Due to the activity of only one enantiomer (L-PZQ) of the racemate in use, tablets are unnecessarily large in size and bad in taste, which leads to decreased compliance especially amongst young patients (Meyer et al., 2009; Talaat and Miller, 1998). In order to overcome these drawbacks, chemical modifications on the PZQ structure were undertaken in recent years, without success (Dong et al., 2010; Liu et al., 2012; Ronketti et al., 2007; Woelfle et al., 2011). A strategy found to be very successful for antimalarial drugs was the synthesis of ferroquine (FQ), a ferrocenyl analogue of the antimalarial drug chloroquine (CQ), as FQ is active on CQ-resistant *Plasmodium falciparum* strains (Dive and Biot, 2008). With this in mind, derivatization of PZQ with ferrocenyl moieties was executed. However, only two ferrocenyl derivatives showed moderate anthelmintic activity against *S. mansoni* in vitro. Therefore an alternative strategy was employed by organometallic derivatization on the aromatic part of PZQ with the help of a $Cr(CO)3$ moiety. An impressive antischistosomal effect was achieved on adult *S. mansoni* in vitro, yet candidates lacked activity in the *S. mansoni* mouse model (Chapter 6).

In this discussion section of my PhD thesis I would like to discuss several important findings and topics in a general and broader manner since objective specific facts have been discussed in the corresponding chapters:

1. Considerations on antischistosomal drug screening
2. Improvements and novel ideas for future antischistosomal drug screening
3. The potential of investigated novel compounds and chemical scaffolds
4. PK studies with antischistosomal drugs

1. Considerations on antischistosomal drug screening

For vigorous antischistosomal drug discovery, an integrated system including rational chemical synthesis and lead optimization, as well as appropriate drug screening strategies is required (Caffrey and Secor, 2011; Geary TG, 2009; Ramirez et al., 2007). To date the phenotypic whole-organism screening with microscopic readout is the core tool of antischistosomal drug research. However it has shortcomings: it is subjective, labor intensive and not very precise (Manneck et al., 2011). Therefore novel readout techniques are needed and are currently being highly investigated (summarized in the introduction) (Caffrey and Secor, 2011).

Although most pharmaceutical R&D departments focus on rational target-based drug discovery, this is only slowly emerging in antischistosomal drug research (Kuntz et al., 2007; Sayed et al., 2008). The target-based approach represents an attractive alternative to whole-organism strategies (Geary TG, 2009), yet the path from target identification to a validated target assay for drug screening needs intensive funding and intense basic research (Renslo and

McKerrow, 2006). In the current situation, the classic phenotypic screen is most likely superior to the target based approach in order to investigate new scaffolds. To note, most marketed drugs were found by phenotypic research (Swinney DC, 2011).

Efforts to identify and improve screening systems have nearly exclusively been undertaken on the schistosomular stage in recent years (Mansour and Bickle, 2010; Paveley et al., 2012; Peak et al., 2010; Smout et al., 2010). At the moment it is the only *Schistosoma* stage, which can be produced in large numbers without the direct need of rodent hosts (Keiser, 2010). Therefore most lead candidates identified result currently from screenings on schistosomula. This might lead to the loss of promising scaffolds, which might present activity on the adult stage. These findings were observed for example for the MMV Box screening undertaken in this PhD thesis, which presented different hit compounds in the schistosomula based screening versus the adult screening. Hence the ideal screening workflow is still under debate (Abdulla et al., 2009; Mansour and Bickle, 2010). If only small sets of compounds are under investigation, screenings on both parasite stages are recommended. Interestingly, only a few years ago, antischistosomal drug screening was based exclusively on adult screening (Ramirez et al., 2007). But at the end of the day, the screening of thousands of compounds has to be feasible and operationally manageable, underlining the continued eligibility of NTS as preliminary screening material. Novel in vitro cultivation system for schistosomes will hardly arise in the near future. Additionally no animals are needed for NTS production following the 3R rule (Brink et al., 1977; Caffrey and Secor, 2011; Keiser, 2010).

2. Improvements and novel ideas for future antischistosomal drug screening

With every investigated compound set, we attempted to optimize our in vitro screening cascade by defining cut-offs for further preclinical testing as well as improving the selection of candidates for in vivo experiments. To note, expected hit-rates are highly dependent on the compound set characteristics (e.g. mode of action). During the course of this work, we observed that primary screens with NTS at a moderate concentration ($\approx 10\ \mu M$) and with an effect determination between schistosomicidal and non-lethal effects (in other words, a live/dead screen) might be more beneficial than dose dependency studies (IC_{50} value determination). The outcome of the two screens would not differ greatly but the throughput would increase. For the adult stage the onset of action seems to represent an important parameter for good in vitro-in vivo correlations. Studies with the MMV box had the advantage that PK profiles of compounds were characterized before in vivo studies were launched. Interestingly the onset of in vitro action clearly correlated with in vivo findings, since only very fast acting drugs presented in vivo activity. Hence, the in vitro-in vivo correlation might be improved by screening potential drug candidates only for short-term exposure (24h) on adult worms instead of the standard 3-day assay. Additionally the PK study of MFQ and EP gave insights into PK-PD correlation. Both compounds possessed extensive half-lives and bioavailability, which made only late in vivo onsets of action possible. This finding underlined the fact that early PK knowledge will help enormously regarding lead characterization.

For the first time it has been shown that *S. haematobium* schistosomula can be used for drug screenings. However the maintenance and procedures are much more labor intense and therefore this assay is only recommended for lead characterization. The use of colorimetric markers for NTS-based drug sensitivity assays had significant limitations, such as high variance in signal, limited number of NTS per well and lack of knowledge of whether metabolic inactivity is essential for antischistosomal drug effect. It was not possible to establish a standardized assay for IC_{50} determination (Master Thesis, Gordana Panic, 2013). As described in another study before, colorimetric readouts might be useful as a yes/ no filter but not for the illustration of dose dependency (Mansour and Bickle, 2010), therefore further investigations should be pursued in future.

No product development partnerships (PDPs) were established in recent years that focused on antischistosomal drug research (Keiser, 2010; Ridley and Kita, 2007). The efforts during this work to establish an ongoing and feasible screening cascade for the development of antischistosomal drugs brought us, amongst others, to the collaboration with MMV. This type of collaboration is in my opinion a very important and fruitful step towards a more productive and faster drug discovery & development. In this way, a diversity of expertise can be gathered and used for this neglected field of drug development. However it should be kept in mind that a second supporting leg, in the form of target based research focusing on MOA insights and resistance development, would scale up antischistosomal drug research.

3. The potential of investigated novel compounds and chemical scaffolds

Arylmethanols

The investigation of MFQ-related arylmethanols underlined the great potential of 4-aminoquinolines as a chemical scaffold, confirmed the moderate activity of 4-phenanthrenmethanols (halofantrine etc.) (Keiser et al., 2009), and introduced the 4-pyridinemethanols as a novel and promising chemical class. One of the two lead candidates, WR7930 possesses mild to phototoxic properties and an extensive half-life ($t_{1/2}$: 25-30 days) in humans (Pullman et al., 1948; Rothe and Jacobus, 1968). These two facts should be strongly considered, since antischistosomal drugs are used mainly in tropical settings and they are used often without prior diagnosis, which requires a very high safety profile of novel candidates. Hence, I would characterize this lead as an interesting chemical scaffold for further studies but abandon it as a lead candidate itself. On the other hand EP has already been used in clinical trials (Cosgriff et al., 1984), which simplifies the next development steps because of possible repurposing. Nonetheless, from my point of view, the therapeutic benefit over MFQ is not large enough to pursue proof of concept studies. First of all, the conducted PK studies detected an extensive half-life of EP in plasma of *S. mansoni* infected mice ($t_{½}$: 7-8 days), which is comparable to the extensive the half-life of MFQ. This fact has to be considered with caution, as slow clearance is not desirable if adverse events occur. Additionally EP did not show great superiority with regard to in in vitro and in vivo activity over MFQ, which makes a potential benefit of EP in humans questionable. Furthermore, the activity of EP on the juvenile stage, representing an essential requirement for novel antischistosomal drug candidates (Keiser and Utzinger, 2007a), needs to be determined first before being able to make a conclusion whether EP represents a promising drug candidate.

Table 1: Stage specific in vivo activities of investigational drugs of interest tested in the framework of this PhD thesis within the *S. mansoni* mouse model. **Red**: lacks activity (WBR <40 %); **Blue**: moderate activity (WBR: 40-80%); **Green**: good activity (WBR>80%), **Grey**: data not available.

Compound	**Dosage**	**Stage specifity of investigational drugs against *S. mansoni* in mice**	
		Juvenile (21 – 28 d)	Adult (35 – 49 d)
Enpiroline	1 x 200 mg/kg		82.7%
WR7930	1 x 100 mg/kg		100.0%
N’-N’-diarylureas	1 x 400 mg/kg		52.5%
2,3-dianilinoquinoxalines	1 x 400 mg/kg		40.8%
Ozonides (OZ418)	1 x 400 mg/kg	100.0%	80.0%
Tetraoxanes	1 x 400 mg/kg	43.1%	75.4%
Tricyclic Peroxides	1 x 400 mg/kg	18.9%	82.8%
3-alkoxy-1, 2-dioxolanes	1 x 400 mg/kg	0.0%	42.5%
Cr-Praziquantel	1 x 400 mg/kg		28.5%

Synthetic Peroxides

The 3-alkoxy-1, 2-dioxolanes do not present as a novel pharmacophor or chemical scaffold, and lack promising in vivo efficacy. In conclusion they should not be prioritized for further preclinical trials.

Regarding the diverse set of OZs, optimized in vivo activities on adult (WBR: 80%) as well as juvenile (WBR: 100%) infections were observed for OZ418 against *S. mansoni* and *S. haematobium*. OZ418 has several important target characteristics of an antischistosomal lead candidate: orally active, stage independent activity and a broad spectrum of activity. Interestingly OZ439, the clinical antimalarial lead candidate, was included in the conducted SAR studies, but lacked good antischistosomal activity. It has been shown before that structural requirements that are mandatory for significant antischistosomal activity, are different from those associated with good antimalarial activity (Xiao et al., 2007). Hence, especially OZ418 might serve as a reasonable drug candidate to promote to further studies. The fact that OZs are already far advanced in clinical trials as antimalarials might even be beneficiary for antischistosomal drug development (Charman et al., 2011). On the other hand the potential risk of developing an antischistosomal drug, which could trigger the resistance development of a corresponding antimalarial drug has to be considered carefully.

The investigation of three new peroxidic classes (tetraoxanes, tricyclic monoperoxides and alphaperoxides) revealed great in vitro activity on the schistosomular stage, but decreased susceptibility on the adult stage. Surprisingly, in vivo we only observed good activity of one tetraoxane and one tricyclic monoperoxides on the adult stage but they lacked activity on the juvenile infection in vivo. Interestingly the most active tetraoxane presented an adamantan residue, as was already observed for the ozonides, which was explained by their improved bioavailability (Vennerstrom et al., 2004). The observation that very good PK profiles are needed for in vivo activity emerged in nearly all projects conducted in this thesis, underlining the importance of early PK studies. Both lead candidates represent new chemical scaffolds (tetraoxanes, tricyclic monoperoxides) but have limitations (they are moderately cytotoxic) as well as being highly investigational drugs. Hence lots of efforts and funding will be needed to overcome these limitations in order to aim for a potent non-toxic tetraoxane or monoperoxidic drug candidate in the near future.

Praziquantel derivatives

The third major chemical class studied in the framework of my PhD thesis represents organometallic derivatives of PZQ. This interesting approach of derivatizing PZQ has not been attempted before. Unfortunately no satisfying in vivo results were observed despite good in vitro performance of the chromium derivatives against adult worms. In summary, this class would have to compete with the parent drug PZQ, offering additional benefits in terms of efficacy, MOA etc. However this is not the case at the moment. Further structural modifications of the active chromium derivatives might lead to improved in vivo activity. Additionally the reason for the in great vitro - in vivo discrepancy should be studied further. Plasma stability has successfully been investigated in metabolic in vitro studies using human liver microsomes (unpublished results). Studying the oral bioavailability of these compounds, with PK profiling and applying alternative administration routes such as i.v. or i.p. could give further insights. Finally, it would be interesting to study the activity of these PZQ derivatives against juvenile *Schistosoma* stages and elucidate a possible MOA expansion compared to PZQ.

N'-N'-diarylureas and 2,3-dianilinoquinoxalines

By screening the open access MMV Box in collaboration with MMV, it was possible to identify two completely novel chemical scaffolds (N'-N'-diarylureas and 2,3-dianininoquinoxalines) as early leads. In comparison to the other lead identification and SAR studies that we conducted, we aimed to elucidate entirely new chemical scaffolds. In recent years well-characterized, novel chemical scaffolds have rarely been presented in antischistosomal drug research. Therefore the moderate in vivo efficacies that were observed with the N'-N'-diarylureas and 2,3-dianininoquinoxalines were satisfactory, since interesting early leads do not have to present all antischistosomal target product profile properties yet (i.e. high in vivo activities following a single oral dose) (Keiser and Utzinger, 2007a). With excellent in vitro activity on adult schistosomes and oral activity in the mouse model, both scaffolds represent exciting starting points for further optimization. However the N'-N' diarylureas class might be more promising, because of its' very easy chemistry and slightly better in vivo effects. Next development steps should involve efforts to elucidate derivatives presenting decreased plasma protein binding in vivo, and to investigate the potential of these classes on juvenile *Schistosoma* infections.

4. PK studies with antischistosomal drugs

Earlier studies documented that the involvement of liver and intestine or genitourinary tract during chronic schistosomiasis infections do alter pharmacokinetics (absorption, distribution, metabolism and elimination

(ADME)) of administered medications (Wilby et al., 2013). Knowledge about the impact of the infection on PK is especially interesting since access to medications in general and antischistosomal treatment is increasing in endemic schistosomiasis regions worldwide (WHO, 2013; Wilby et al., 2013). Studies with PZQ reported increased AUCs and c_{max} as well as slower clearance, resulting in altered clinical effects such as an increase in adverse side effects (el Guiniady et al., 1994; Mandour et al., 1990; Watt et al., 1988; Wilby et al., 2013). PK parameter changes of PZQ seemed to be in line with the stage of hepatic insufficiency (el Guiniady et al., 1994). Similar patterns were observed within our PK studies of MFQ and EP, both drugs showed extensive half-lives and AUCs, presenting slow drug clearance. The late onset of action of EP and MFQ was in line with the long half-life of both drugs. Probably, a long time period of drug exposure in the infected animals is necessary for EP and MFQ to unfold their activity instead of reaching high c_{max} levels, hinting to an AUC dependent in vivo activity.

Especially co-endemic regions should be considered for thorough PK studies, since decreased drug clearance of administered antimalarials caused by schistosomiasis infections can have great impact on the clinical outcome, such as resistance development and adverse effects. To date only limited numbers of studies have been conducted with small numbers of patients enrolled. Knowledge about the PK-PD relationships would improve antischistosomal drug discovery (Wilby et al., 2013). The next step would be to conduct population based PK studies, which might increase our knowledge on the employed chemotherapy. PK studies on anthelmintics in infected individuals are especially interesting since target groups are mainly children, which possess an altered metabolism. As antischistosomal drug discovery gains more focus and funding, promising compounds will begin accumulating at the very early stages of drug discovery. Hence, drug development should particularly aim to fund resource-intensive lead-optimization chemistry as well as in vivo PK studies (Renslo and McKerrow, 2006).

2 Conclusion

This thesis aimed to accelerate antischistosomal drug discovery by improving and implementing a solid screening cascade and by boosting the antischistosomal drug pipeline with new candidates and chemical scaffolds.

The following improvements to the screening process were achieved. Activity observed following short-term in vitro drug exposure on adult schistosomes was found to be the best in vitro parameter correlating with in vivo outcome. Primary schistosomula screenings were confirmed as very useful however, due to varying stage susceptibilities, the definition of cutoffs has to be considered carefully in order to assure balanced hit-rates on the adult secondary screening. *S. haematobium* schistosomula were successfully introduced as potential drug screening material, but no alternative readouts in terms of colorimetric markers showed promising results.

The second major aim of the thesis was to identify and characterize new antischistosomal lead candidates and chemical scaffolds. Overall compounds with antimalarial activity have proven to be promising and fruitful starting points for antischistosomal drug discovery. Amongst all antimalarials, synthetic peroxides and praziquantel derivatives tested, EP (4-pyridinemethanol) and OZ418 (ozonide) presented the most potential and fulfilled the most requirements for promising drug lead candidates. However EP has to prove superior efficacy or drug properties over MFQ before further clinical assessment should be launched. The use of both compounds as antischistosomal drugs must always be carefully considered in terms of possible consequences for antimalarial treatment. The 4-quinolinemethanols were proven to interact with the hemoglobin pathway of schistosomes. The main mechanism of activation for synthetic peroxides was shown to be most likely iron independent. Further studies should be launched to investigate the mechanism of action in more detail. Two entirely new scaffolds, the N'-N'-diaryureas and 2,3-dianilinoquinoxalines, were identified and further lead optimization studies should be launched .

Importantly the extensive impact of schistosomiasis infections on drug profiles was presented for EP and MFQ. Chronic *S. mansoni* infections led to slowed in vivo drug clearance. Hence, additional PK studies are warranted and would offer new opportunities for a highly interesting research area.

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Chapter 8

Appendix

During the course of this thesis contributions were additionally made to the following manuscripts.

8.1 Antiparasitic drugs for pediatrics: systematic review, formulations, pharmacokinetics, safety, efficacy and implications for control.

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Antiparasitic drugs for paediatrics: systematic review, formulations, pharmacokinetics, safety, efficacy and implications for control

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SUMMARY

Drug development for paediatric applications entails a number of challenges, such as the wide age spectrum covered – from birth to adolescence – and developmental changes in physiology during biological maturation that influence the efficacy and toxicity of drugs. Safe and efficacious antiparasitic drugs for children are of pivotal importance given the large proportion of burden attributable to parasitic diseases in this age group, and growing efforts to administer, as widely as possible, antiparasitic drugs to at-risk populations, such as infants and school-aged children, often without prior diagnosis. The purpose of this review is to investigate whether antiparasitic drugs have been adequately studied for use in paediatrics. We approached this issue through a systematic review using PubMed and the Cochrane Central Register of Trials covering a period of 10 years and 8 months until the end of August 2010 to identify trials that investigated efficacy, safety and pharmacokinetic (PK) parameters of antiparasitic drugs for paediatrics. Overall, 269 clinical drug trials and 17 PK studies met our inclusion criteria. Antimalarial drugs were the most commonly studied medicines (82·6%). Most trials were carried out in Africa and children aged 2–11 years were the age group most often investigated. Additionally, we critically examined available drug formulations for anthelminthics and identified a number of shortcomings that are discussed. Finally, we shed new light on current proposals to expand 'preventive chemotherapy' to preschool-aged children and emphasise that new research, including risk-benefit analyses, are needed before such a strategy can be adopted more widely.

Key words: Antiparasitic drugs, antimalarials, anthelminthics, preventive chemotherapy, paediatrics, infants, children, adolescence, systematic review, drug formulation.

INTRODUCTION

Drug development for the paediatric population is a challenging endeavour, since a wide age spectrum from birth to adolescence is covered. A useful stratification of the paediatric population is the one proposed by the World Health Organization (WHO) using the following five classes: (1) preterm newborn infants; (2) term newborn infants (0 to 28 days); (3) infants and toddlers (>28 days to 23 months); (4) children (2 to 11 years); and (5) adolescents (12 to 16–18 years) (WHO, 2007*a*). Importantly, the developmental changes in physiology during biological maturation from newborns to adolescence influence the efficacy and toxicity of drugs. Indeed absorption, distribution, metabolism, excretion and toxicity (ADMET; see Glossary) are all age dependent. Textbooks and detailed reviews are available that summarise key factors responsible for differences in drug disposition between paediatric and adult population such as proportions of body fat, protein, extracellular water, organ size, membrane permeability, plasma proteins, enzymes, glomerular filtration or tubular secretion (Strolin Benedetti *et al*. 2005; Rakhmanina and van den Anker, 2009). For example, body water is decreased from 80% in newborns to 60% in 5-month-old infants (WHO, 2007*a*). Moreover, absorption, stability and ionisation of drugs depend on gastric pH, which is age-dependent. In the neonatal period an elevated pH is observed (i.e. the pH is neutral rather than acidic as in adults), which explains that, in younger age groups, a greater bioavailability of acid-labile compounds occurs (e.g. penicillin) (Kearns *et al*. 2003).

In view of the aforementioned issues it is not surprising that the need for age-appropriate pharmacotherapy was already recognised more than 100 years ago (Kearns *et al*. 2003). The International Conference on Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH-E-11) states that "Paediatric

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patients should be given medicines that have been appropriately evaluated for their use" (http://www.fda.gov). We pose the question whether antiparasitic drugs have been adequately evaluated prior to wider application in paediatric populations. Indeed, addressing this question served as the main motivation for the current paper, which forms part of a special issue of *Parasitology* pertaining to "Progress in Paediatric Parasitology" (see Stothard and Booth editorial in this special issue).

We first provide a short historical background on paediatric drug development and highlight practical, ethical and economic issues. We then juxtapose the latest United Nations (UN) population figures and disease burden estimates in the lowest income countries. To strengthen the current evidence-base of antiparasitic drugs in the paediatric population, we performed a systematic review using two readily available electronic databases. We examined the type of drugs investigated, the age of the study participants and performed a temporal and geographical analysis of studies meeting our inclusion criteria. Paediatric pharmacokinetic (PK) studies were also examined. With an emphasis on anthelminthic drugs, we critically reviewed available drug formulations. Finally, in the current era of 'preventive (anthelminthic) chemotherapy' (see Glossary), that is the regular administration of antiparasitic drugs to entire at-risk populations (WHO, 2006, 2010*a*), we discuss implications for control programmes that aim at including also preschool-aged children.

PAEDIATRIC DRUG DEVELOPMENT

Regulatory, ethical, commercial and economic issues

Regulatory efforts to protect children from harmful medications began in the mid-20th century, in response to serious adverse events, such as limb malformations caused by thalidomide (Barsch and Otte, 2010). Indeed, thalidomide (Contergan®), which had been used as a treatment for recurring morning sickness in pregnant women during the 1950s and the early 1960s, caused peripheral neuritis and malformations, e.g. phocomelia, in babies of mothers who had taken thalidomide over the course of their pregnancies (Stötter, 2007; Barsch and Otte, 2010). Once the evidence of these serious adverse events of thalidomide had been established through case-control studies, the medication was withdrawn from the market (Mellin and Katzenstein, 1962). As a consequence, many drugs received marketing authorisation for use in adults only. However, an increase in off-label use (see Glossary) was subsequently observed, amounting to 80% in paediatric patients (Pandolfini and Bonati, 2005; Stötter, 2007).

Bearing the aforementioned dilemmas in mind, over the past two decades, regulations have been enacted upon and incentives created; first in the US (e.g. Food and Drug Administration Modernization Act (FDAMA) instigated in 1998 and Best Pharmaceuticals for Children Act (BPCA) put forth in 2002; see Glossary) and, second by a number of EU paediatric guidelines (Rose, 2009). The purpose of these regulations was to improve paediatric drug development, particularly in terms of safety (Schachter and Ramoni, 2007; Macleod, 2010). It is clear that these initiatives and regulations have changed the landscape for paediatric drug development. For example, there are recent signs of enhanced research activities going hand-in-hand with clinical trials on the efficacy and safety of drugs in children including PK studies and the development of drug formulations that are suitable for the paediatric population (Macleod, 2010).

Nonetheless, there are a number of challenges regarding clinical trials in children. Ethical issues include the complexity to obtain written informed consent from parents and legal guardians, as well as assents from participating children (Kuepfer and Burri, 2009). Furthermore, the level of invasiveness should be kept as little as possible, and hence the number of blood samples and the amount of blood taken in PK studies should be minimised (Howie, 2011). Scientific issues comprise, for example, the necessity to stratify the patient population into different age groups or the need to develop microassays to analyse small amounts of biological samples that are typically obtained from paediatric populations (Conroy *et al*. 2000; WHO, 2007*a*; Choonara, 2009).

Finally, there are commercial and economic issues as the market for paediatric medicines is small compared to the adult population (estimated to be less than 10% of the total prescription drug market (Milne, 2009)), and hence lacks attractiveness for the international pharmaceutical industry. While blockbusters mainly address highly prevalent chronic diseases, children often experience acute illnesses, and the chronic conditions that do exist in the paediatric population are mostly rare disorders (WHO, 2007*b*; Milne and Bruss, 2008).

PAEDIATRIC DRUG DEVELOPMENT, DEMOGRAPHY AND DISEASE BURDEN

In the US, since the FDAMA institutionalised an incentive of a 6-month patent extension for previously approved drugs in July 1998, a total of 173 drugs were granted paediatric exclusivity (http://www.fda.gov/downloads/Drugs/DevelopmentApprovalProcess/DevelopmentResources/UCM223058.pdf). These developments, it was claimed, showed the achievements made by FDAMA for the paediatric population. However, it should be noted that most of these patent extensions were for drugs targeting the central nervous system (CNS) (e.g. anti-depressants), cardiovascular system (e.g. ACE inhibitors), alimentary tract and metabolism (e.g. anti-diabetic drugs) and anti-infectives (e.g. antibiotics). Indeed, a deeper analysis revealed a major discrepancy between drugs granted exclusivity and those frequently used by children (e.g. respiratory drugs, dermatologicals and anti-infectives) (Boots *et al*. 2007). Hence, the real needs in paediatric drug development and use have not been addressed by exclusivity rights granted by FDAMA. In contrast they were primarily driven by the adult drug market (Boots *et al*. 2007).

Those drugs that are currently available and are essential for children must be prioritised for paediatric development. Indeed, there is growing recognition that among essential medicines, issues of safety, access and formulations for the paediatric population are of pressing global public health relevance. This can be further underscored by juxtaposing population figures put forth by the UN (United Nations, 2009). In 2010, an estimated 2·47 billion were newborns, infants, children and adolescents below the age of 19 years. The paediatric population, therefore, currently accounts for more than a third (35·7%) of the world's population. Meanwhile, it has been estimated that 432·5 million children and adolescents currently live in the least developed countries, which comprise 49 countries, of which 33 are in Africa, 10 in Asia, five in Oceania and one in Latin America and the Caribbean. Compared to the total population in these 49 countries (i.e. 854 million), the paediatric population therefore accounts for more than 50% (United Nations, 2009).

It is encouraging to note that new campaigns have been launched with the goal to expedite paediatric drug development. For example, "Make medicines child size" is a global initiative launched in December 2007 under the leadership of WHO in order "to raise awareness and accelerate action to address the need for improved availability and access to safe child-specific medicines for all children under 12" (http://www.who.int/childmedicines/en/). High priority therapeutic areas include respiratory diseases as well as parasitic and infectious diseases. The importance of the latter group of diseases must be emphasised, which is clear when one examines global burden of disease estimates. In 2004, for example, children aged below 14 years experienced the loss of 548·3 million disability-adjusted life years (DALYs; see Glossary). Three-quarter of this burden (409·8 million DALYs) occurred among children from low-income countries. Infectious and parasitic diseases were responsible for more than 300 million DALYs, with 79% of this burden concentrated in low-income countries, disproportionally shared by children younger than 14 years (58%) (WHO, 2008*a*). The proportion of disease burden attributable to infectious and parasitic diseases in children in low-income countries was 38% (154·3 million DALYs) (WHO, 2008*a*). Moreover, infectious diseases are a leading cause of death in children younger than 5 years, particularly in low-income countries. The latest estimates for the year 2008 suggest that among the 8·80 million deaths in children under the age of 5 years, two-third (68% or 5·97 million deaths) were due to infectious diseases, the three most important of which were pneumonia (1·56 million deaths), diarrhoea (1·34 million deaths) and malaria (732,000 deaths) (Black *et al*. 2010). Of note, intestinal parasites are responsible for reductions in appetite, absorption, digestion and increasing intestinal nutrient losses (Lunn and Northrop-Clewes, 1993), and hence might contribute to undernutrition, which is the underlying cause of a third of deaths in children younger than 5 years (Black *et al*. 2010).

CLINICAL TRIALS WITH ANTIPARASITIC DRUGS FOR PAEDIATRICS

Search strategy and selection criteria of a systematic review

We were interested in clinical trials involving antiparasitic drugs in the paediatric population, as well as PK studies, covering a period of 10 years and 8 months until the end of August 2010. Previous articles highlighted the paucity of paediatric clinical trials involving children in the developing world (Sammons and Choonara, 2005; Nor Aripin *et al*. 2010*a*,*b*). For example, in 2007, only one out of four trials were carried out in developing countries (Nor Aripin *et al*. 2010*b*). Between 1996 and 2002, a total of 99 trials carried out in the developing world were identified with a specific focus on antiparasitic and anti-infective drugs. However, no details were presented on the type of medication studied or age group involved (Nor Aripin *et al*. 2010*a*).

We performed a systematic review on PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and the Cochrane Central Register of Controlled Clinical Trials (http://onlinelibrary.wiley.com/o/cochrane/cochrane_clcentral_articles_fs.html). In order to maximise sensitivity and specificity (Kastner *et al*. 2006), we used the following age-specific MeSH terms: 'child', 'adolescent' or 'infant', in combination with 'clinical trial' and 'antiparasitic agent'. For the search on PK studies, the same age-specific MeSH terms were employed, but in combination with 'pharmacokinetics'. Our searches were temporally restricted (from 2000 to the end of August 2010), while there was no language restriction. Abstracts of the retrieved publications were analysed, adhering to a standard protocol developed by the authors. Studies had to pass the following inclusion criteria: (1) study population of children or adolescents (age: ⩽18 years); (2) trials investigating drug efficacy (e.g. no vaccination or supplements); and (3) study performed on any parasitic disease. The following information was retrieved from each study that met our inclusion criteria: (i) year of publication; (ii) parasite studied; (iii) study setting and country;

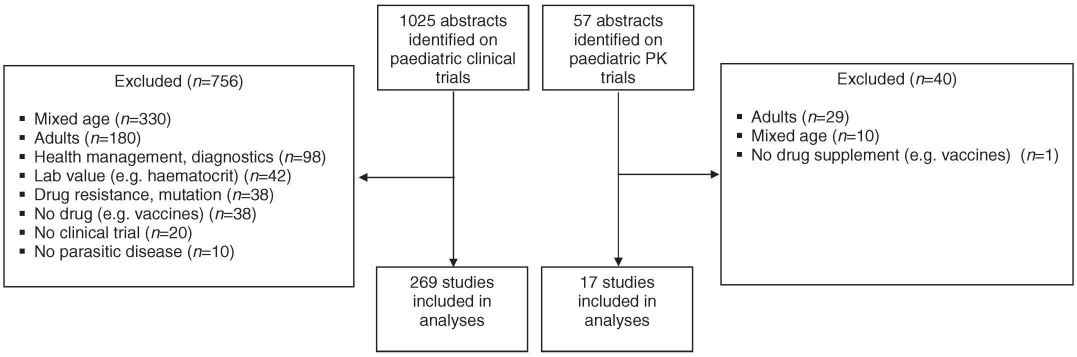


Fig. 1. Flow chart of study selection for systematic review of clinical trials and PK studies with antiparasitic drugs in children covering a period of 10 years and 8 months until the end of August 2010.

(iv) sample size; (v) age and age group of study population; (vi) methodological quality of trial; and (vii) type of medication used (e.g. substance, preparation and Anatomical Therapeutical Chemical (ATC) classification system; see Glossary).

Number of studies identified and age groups

A total of 1025 articles were identified. Based on the abstracts 756 studies were excluded and full-text copies were obtained from 269 articles. The three main reasons for exclusion were: (1) study population consisting of children and adult patients (44%); (2) entire study population aged 18 years and above (24%); and (3) trials investigating diagnostic tests or focusing on health management issues (13%). Additional exclusion criteria are summarised in Fig. 1.

In four out of five trials (80%) both infants and children (113 trials) or children only (102 trials) were included. The remaining 20% of the trials involved the following age classes: (1) children and adolescents (34 trials); (2) infants (15 trials); (3) infants, children and adolescents (four trials); and (4) adolescents (one trial). More than 90% of the trials enrolled children (93·7%), while only 39 trials included adolescents. The number of participants per trial ranged from 10 to 4906. One third of all trials recruited between 101 and 250 participants. Table 1 shows the frequency of trials, stratified by different sample sizes.

Outcomes from temporal and geographical analyses

Our temporal analysis revealed that the number of paediatric drug trials steadily increased between 2000 (only two trials) and 2004 (37 trials), decreased in the following 2 years (25 and 23 studies in 2005 and 2006, respectively), and subsequently varied between 27 and 36 per year. In 2010, until the end of August 2010, a total of eight trials were identified for antiparasitic drugs in paediatrics (Fig. 2).

The studies examined were carried out in 61 countries. More than a third of the trials (38·7%) were conducted in only five countries: Thailand (24 trials), Nigeria (21 trials), United Republic of

Table 1. Number of paediatric patients involved in clinical drug trials and pharmacokinetic (PK) studies identified through a systematic review covering a 10-year period until the end of August 2010

	Clinical drug trials		PK studies	
Sample size (n)	No.	%	No.	%
⩽ 50	32	11·9	11	64·7
51–100	40	14·9	3	17·6
101–250	89	33·1	2	11·8
251–500	48	17·8	0	0
501–1000	32	11·9	1	5·9
1001–2500	26	9·7	0	0
2501–5000	2	0·7	0	0
Total	269	100	17	100

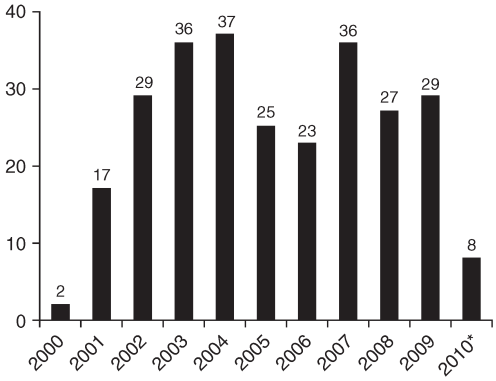


Fig. 2. Temporal analysis of paediatric drug trials identified through a systematic review covering a 10-year period until August 2010. (* indicates that in 2010 only the first 8 months were analysed).

Tanzania (21 trials), Uganda (20 trials) and Ghana (18 trials). Interestingly, almost three-quarter of the trials were implemented in Africa (72·7%). The respective percentage of trials carried out in Asia, South America, North America, Australia and Europe was 16·8%, 3·6%, 3·3%, 2·0% and 1·6%, respectively (Fig. 3).

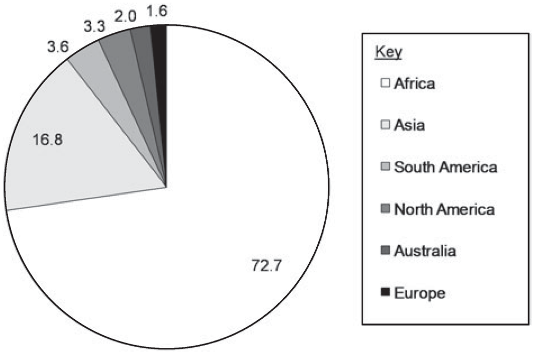


Fig. 3. Geographic location of paediatric drug trials (%) identified through a systematic review covering a period of 10 years and 8 months until the end of August 2010.

Drugs investigated

Ninety-three percent of the drug trials that we identified through our systematic review investigated antiparasitic medications. The remainder of the studies (7%) examined drugs of the following ATC classes: alimentary tract and metabolism, blood and blood forming organs, dermatologicals, systemic hormonal preparations, anti-infectives for systemic use, antineoplastic and immunomodulating agents, musculo-skeletal system, CNS and respiratory system. Less than a third (29%) of all included studies were classified as randomised controlled trials.

Fig. 4 shows that most of the antiparasitic drugs studied were antimalarials (82·6%), followed by therapies for gastrointestinal nematodes (9·8%), amoebiasis and other intestinal protozoal infections (3·7%), schistosomiasis (2·9%) and agents against leishmaniasis and trypanosomiasis (1·0%). A closer inspection of the drugs studied showed that sulphadoxine-pyrimethamine (SP) was the most frequently trialled medication (20% of all studies), which is explained by the use of SP as part of malaria control, with a strategy termed intermittent preventive therapy in infants (IPT$_i$) and children (IPT$_c$) (see Glossary) (Aponte *et al*. 2009; Gosling *et al*. 2010). Other frequently used antimalarials in the paediatric population identified through our systematic review were amodiaquine and artesunate (both 15%) and chloroquine (10%). Albendazole was the most commonly studied anthelminthic drug (5% of all trials).

PK studies

Fifty-seven studies were retrieved after systematically searching for PK investigations of antiparasitic drugs in paediatrics covering a period of 10 years and 8 months until the end of August 2010. Forty studies were excluded when applying our selection criteria (study participants aged $\geqslant$18 years or studies including both adults and children ($n = 39$); one trial investigated a supplement rather than a drug).

The sample size of the 17 included studies ranged from as few as 10 individuals to 899 subjects (Table 1). Two-third of the trials ($n = 11$) recruited 10 to 50 participants, while only one trial had a sample size of more than 250 individuals. The most commonly studied age group were children: 10 trials were performed exclusively with children and five studies included both children and infants. The age groups of solely infants and adolescents in combination with children were only represented in a single study each. PK parameters of antimalarial drugs were studied in 15 of the 17 trials. The remaining two PK studies investigated anthelminthics and a neoplastic agent for leishmaniasis. The antimalarial drugs artesunate and quinine were investigated most often (each drug in 29% of the PK studies identified). With regard to anthelminthic drugs, albendazole and praziquantel were the only compounds subjected to PK investigations in the paediatric population.

DRUG FORMULATIONS

General considerations

Although there is a pressing need for developing new antiparasitics, very few drugs have been marked-approved in recent years. For example, while 11 new antimalarials have been marketed between 2000 and 2009, no new drugs have been approved in any of the other parasitic disease categories over the same period (Cohen *et al*. 2010). Given the paucity of clinical trials in paediatrics focusing on diseases other than malaria, it is conceivable that only very few, if any, drugs for other parasitic diseases will be forthcoming in the near future. However, drugs can be considerably optimised when new drug formulations are developed (Kayser *et al*. 2003).

Desired criteria for drug formulations include (1) good bioavailability; (2) safe excipients and ingredients; (3) dose uniformity; (4) ease and safety of administration; and (5) socio-cultural acceptability (Breitkreutz and Boos, 2007). Based on factors such as solubility and taste of the compound formulation, development can be a time consuming and technically challenging task and the financial implications are considerable (Milne, 2009). Yet, an acceptable formulation is particularly important for children. Indeed, one of the key issues in the development of paediatric drugs is the selection of the most appropriate dosage form in relation to age. Numerous drug

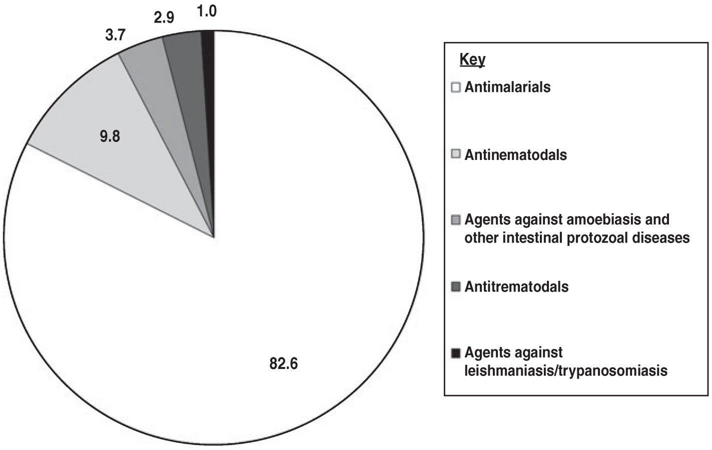
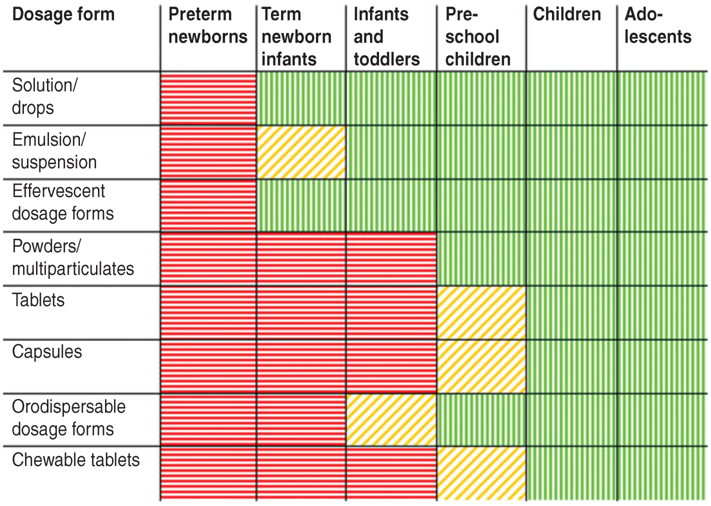


Fig. 4. Drug classes investigated (%) in a systematic review covering a period of 10 years and 8 months until the end of August 2010.

administration routes are available, such as peroral, nasal, parenteral, topical, rectal or buccal (Breitkreutz *et al*. 2007). Matrixes combining different paediatric age groups, routes of administration and dosage forms have therefore been developed to assist in selecting the ideal formulation (see, for example, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003782.pdf and Breitkreutz (2009)). A slightly simplified matrix for oral routes of drug administration using 'traffic light colours' is depicted in Fig. 5. Ideal, recommended and desired dosage forms are shown using green colour, less preferred but probable applicable dosage forms are marked with orange colour and not applicable oral dosage forms are depicted in red. For example, solid dosage forms might pose problems to small children as they have difficulties swallowing tablets. It follows that tablets and capsules are not recommended for infants and toddlers, and applicable but not preferred for preschool-aged children.

Clearly, oral drug administration is the predominant route in paediatric patients and different oral dosage forms (e.g. solutions, syrups, powder, granules and effervescent tablets) have been developed. However, in particular when drugs are not licensed for children (i.e. off-label use) suitable liquid forms are often not available. Inert ingredients must be selected very carefully as children might have adverse reactions to colourings, flavouring or preservatives, which are commonly used in adult formulations (Milne, 2009). Taste is a particularly crucial issue to ascertain high compliance in children. For example, a survey carried out in the US interviewing 500 parents



Dosage form	Preterm newborns	Term newborn infants	Infants and toddlers	Pre-school children	Children	Ado-lescents
Solution/ drops	🟥	🟩	🟩	🟩	🟩	🟩
Emulsion/ suspension	🟥	🟨	🟩	🟩	🟩	🟩
Effervescent dosage forms	🟥	🟩	🟩	🟩	🟩	🟩
Powders/ multiparticulates	🟥	🟥	🟥	🟩	🟩	🟩
Tablets	🟥	🟥	🟥	🟨	🟩	🟩
Capsules	🟥	🟥	🟥	🟨	🟩	🟩
Orodispersable dosage forms	🟥	🟥	🟨	🟩	🟩	🟩
Chewable tablets	🟥	🟥	🟥	🟨	🟩	🟩

Fig. 5. Matrix combining different dosage forms for oral routes of drug administration for different paediatric age groups.

Table 2. Available drug formulations, their producers, recommended dosages and paediatric use for drugs used for the treatment of schistosomiasis, food-borne trematodiases, soil-transmitted helminthiases and strongyloidiasis

Infection	Drug	Formulation	Producer(s)	Recommended dosage	Paediatric use
Schistosomiasis and food-borne trematodiases	Praziquantel	Tablet, 600 mg	Four large scale producers: Merck, Shin Poong, EIPICO, Cipla	40 mg/kg once (schistosomiasis), multiple doses (schedule depending on trematode; food-borne trematodiases)	Experience in children below 4 years is limited
	Praziquantel	Syrup, 120 mg/ml (Epiquantel)	EIPICO		
Fascioliasis	Triclabendazole	Tablet, 250 mg	Novartis	10 mg/kg once (20 mg/kg in the case of treatment failures)	No experience in children below 6 years
Soil-transmitted helminthiases	Albendazole	Chewable tablet, 200 and 400 mg	GlaxoSmithKline, many generic producers	400 mg once for individuals aged 2 years and above, single 200 mg once for individuals aged 1–2 years	No experience in children below 1 year
		Suspension, 100 mg/5 ml		20 ml once for individuals aged 2 years and above, 10 ml once for individuals aged 1–2 years	
	Mebendazole	Chewable tablet, 100 and 500 mg	Janssen Pharmaceutica, many generic producers	500 mg once, 100 mg twice a day for 3 days	No experience in children below 1 year
		Suspension, 100 mg/5 ml		25 ml once, 5 ml twice a day for 3 days	
	Levamisole	Tablet, 50 and 150 mg	ICI. Pharmaceuticals, many generic producers	2·5 mg/kg once	No experience in children below 1 year
		Suspension, 40 mg/5 ml	Generic producers (e.g. EIPICO)	2·5 mg/kg once	
	Pyrantel pamoate	Chewable tablet, 250 mg	Pfizer	10 mg/kg once, 10 mg/kg for 3 days for individuals aged 1 year and above	No experience in children below 1 year
		Suspension, 50 mg/ml	Pfizer	1 ml for every 5 kg of body weight	
Strongyloidiasis	Ivermectin	Tablet, 3 and 6 mg		200 μg/kg single dose 15 kg and above	Safety and efficacy in individuals weighing less than 15 kg have not been established
	Albendazole	See above	See above	Multiple doses (e.g. 400 mg twice daily for 3 days) ages 2 years and above	See above

and caretakers has shown that taste was, among two-third of the children, a reason for non-compliance (Milne and Bruss, 2008; Milne, 2009).

Anthelminthic drug formulations

Table 2 shows the most important anthelminthic drugs currently employed within the frame of preventive chemotherapy programmes, including the most common formulations (WHO, 2006; Hotez *et al*. 2007). Importantly, these anthelminthics have become the drugs of choice against schistosomiasis, food-borne trematodiases and soil-transmitted helminthiases, the latter including strongyloidiasis. Indeed, millions of people are given one or several of these drugs each year, particularly school-aged children (WHO, 2008*b*, 2010*b*,*c*; Fenwick *et al*. 2009). Manufacturers and recommended dosages, both for adults and the paediatric population are presented. In the remainder of this section we highlight shortcomings linked to drug formulations available for major helminthiases, such as dosing accuracy, lack of PK investigations, unknown bioavailability for extemporaneous preparation and indeed lack of suitable formulations for small children.

Praziquantel

In 2008, 17·5 million individuals were treated with praziquantel in the frame of preventive chemotherapy programmes against schistosomiasis (WHO, 2010*b*). It is conceivable that several more million people were administered praziquantel, facilitated by local, regional and national control efforts, purchase from pharmaceutical stores, distributed by non-governmental organisations and aid workers, but these data were not reported to WHO. However, tens of millions of individuals, particularly school-aged children at-risk of schistosomiasis in Africa, still lack access to praziquantel (Utzinger *et al*. 2009; Hotez *et al*. 2010; WHO, 2010*b*). There is a paucity of data regarding the number of people at risk of food-borne trematodiases who have been treated with praziquantel in the frame of preventive chemotherapy.

At present, praziquantel is recommended for individuals aged 4 years and above (WHO, 2002*a*; Biltricide, package insert). Praziquantel tablets (600 mg) are large, bitter in taste and the recommended dosage for preventive chemotherapy is 40 mg/kg, administered in a single oral dose (Hotez *et al*. 2007; Meyer *et al*. 2009). Praziquantel tablets are often split into two or even four parts. However, it has been shown that the weight of split tablets ranges from 50–150% of the desired weight of the half-tablet weight and even the use of tablet cutters do not improve accuracy (Standing and Tuleu, 2005). Furthermore, although crushing of praziquantel tablets has been recommended by the manufacturer (package leaflet; http://www.merck.com), and indeed employed in recent studies with preschool-aged children infected with schistosomes (Odogwu *et al*. 2006; Betson *et al*. 2010; Garba *et al*. 2010), the bioavailability of the crushed formulation might differ from the original tablet form. To our knowledge, the bioequivalence of praziquantel administered as crushed tablets *versus* tablets swallowed as a whole remains to be investigated. For example, a recent study, which compared PK parameters of telithromycin administered either as whole tablets or crushed (and offered with nutritional supplement drink) found that both methods of administration were bioequivalent. Hence, crushing of telithromycin could be a viable method of administration for patients unable to swallow whole tablets (Lippert *et al*. 2005). However, it should be kept in mind that it is difficult to mask the bitter taste of crushed tablets. Infants and children react unfavourably to bitter tastes and the more bitter the drug, the more likely it will be rejected (Mennella and Beauchamp, 2008; Schwartz *et al*. 2009). Since praziquantel is marketed as racemate, and the antischistosomal activity is stereoselective, new efforts are underway to develop a low-cost chemical synthesis for the active enantiomer, L-praziquantel (Meyer *et al*. 2009). Of note, L-praziquantel is less bitter than racemic praziquantel (Meyer *et al*. 2009) and one study in the People's Republic of China reported less adverse events of L-praziquantel compared to the racemate (Wu *et al*. 1991).

A praziquantel syrup formulation is available (Doenhoff *et al*. 2009). However, it is not commonly used and not known whether bioequivalence has been thoroughly studied. In addition, it is not known which excipients, which are required for dose uniformity, stability or taste (Standing and Tuleu, 2005) are present. A detailed summary of excipients, their technological function and adverse events has been provided by Pifferi and Restani (2003).

Finally, there is a need to study the safety, dose-response and PK of praziquantel thoroughly in children below the age of 4 years, because recent studies in different epidemiological studies documented that schistosomiasis can occur in infants and preschool-aged children, hence well before the age of 4 years (Mafiana *et al*. 2003; Bosompem *et al*. 2004; Odogwu *et al*. 2006; Stothard and Gabrielli, 2007; Betson *et al*. 2010; Garba *et al*. 2010; Sousa-Figueiredo *et al*. 2010*b*; Stothard *et al*. 2011). Preschool-aged children are presently not targeted in schistosomiasis preventive chemotherapy campaigns. However, it has been emphasised that preschool children do not only play a role in local disease transmission, but importantly active infections acquired at early ages might aggravate the clinical significance of the disease in later-life (see Stothard *et al*. in this special issue). First trials have been carried out, treating preschool children with half or three quarters of a tablet depending on height, using an extended dose pole (Sousa-Figueiredo *et al*. 2010*a*,*b*). However, as highlighted in the present manuscript, a century ago it was recognised by Dr. Jacobi that children and infants are not miniature men and women requiring just reduced doses (Kearns *et al*. 2003). Rather than using an empirical approach and applying weight-based calculations to the adult dose, there is a need for in-depth studies with praziquantel, including PK, to determine the proper formulations and doses in paediatric patients.

Triclabendazole

Similar to praziquantel, triclabendazole is currently not registered for use in young children (< 6 years of age), but off-label use in this age group has been reported (WHO, 2007*b*). Triclabendazole is the current drug of choice against fascioliasis (Keiser *et al*. 2005; Fairweather, 2009; Keiser and Utzinger, 2010) and we strongly recommend that available data of the safety and efficacy of triclabendazole in preschool-aged children be scrutinized. We anticipate that the evidence-base is currently insufficient, and hence additional clinical trials are warranted to fill existing gaps. Furthermore, an appropriate triclabendazole formulation for young children might be considered. However, it is unlikely that progress in this field will be made in the near future given the fact that fascioliasis is one of the most neglected tropical disease and triclabendazole is currently registered in only four countries (Keiser *et al*. 2005).

Albendazole, mebendazole, levamisole and pyrantel pamoate

The main drugs used for the treatment of soil-transmitted helminthiases are albendazole, mebendazole, levamisole and pyrantel pamoate (Bethony *et al*. 2006; Keiser and Utzinger, 2008). Clearly, the former two – the benzimidazoles albendazole and mebendazole – are the most widely used drugs within the frame of preventive chemotherapy campaigns (WHO, 2006; Hotez *et al*. 2007). At the moment all four drugs are recommended for children aged 12 months and above. There are currently insufficient data on safety and efficacy for use in younger children which is a problem, as infections are often acquired by infants before they reach 12 months (WHO, 2002*a*; Montresor *et al*. 2003). It is interesting to note that for the two widely used benzimidazoles no age-specific dosing regimens exist. For example, a 1-year-old child (approximately 10 kg) receives the same 500 mg mebendazole dose than an 80 kg adult. Indeed, dosage adaptations based on weight or body surface area (Kearns *et al*. 2003) have not been suggested for the benzimidazoles in children. To our knowledge PK studies with these drugs have neither been undertaken in infants nor in children. We and others have highlighted that the majority of anthelminthics currently used for preventive chemotherapy have been developed in veterinary parasitology, and hence were not sufficiently optimised for treating human helminth infections (Geary *et al*. 2010; Keiser and Utzinger, 2010).

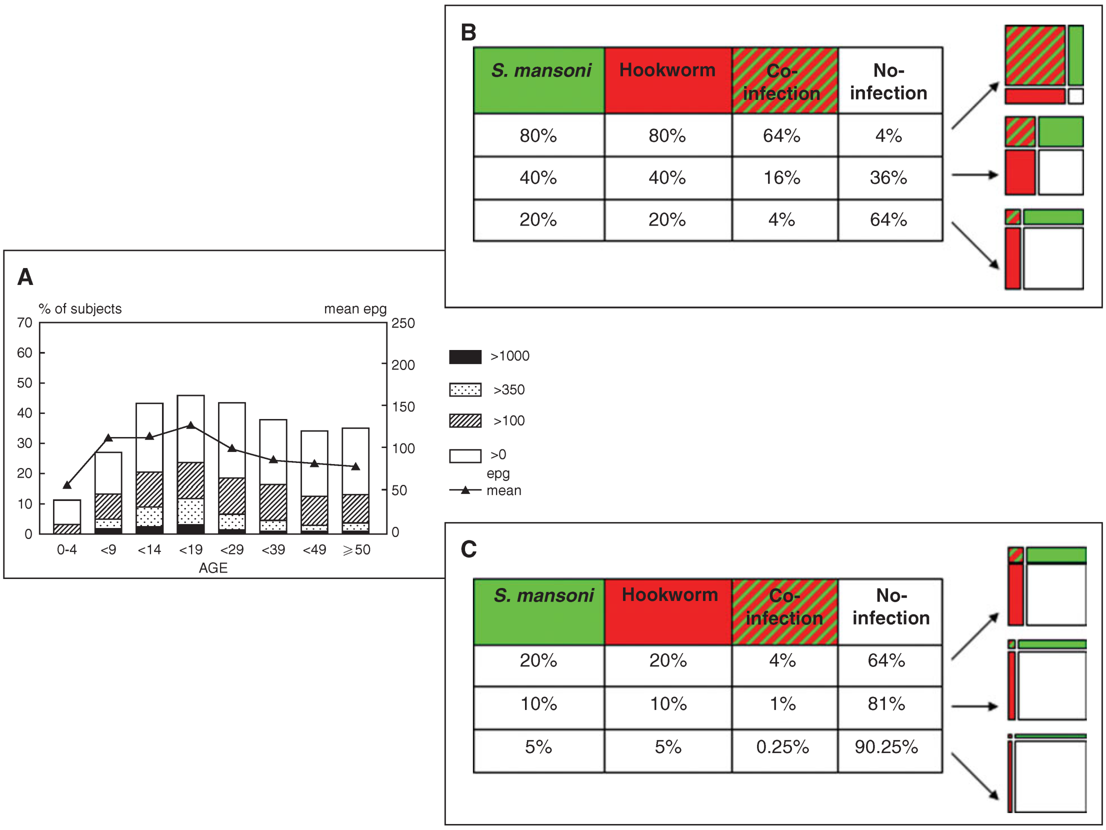
Albendazole, mebendazole, levamisole and pyrantel pamoate are available as liquid formulations, which are obviously most appropriate for paediatric patients (Table 2). Typical target dose volumes for paediatric liquid formulations are <5 ml for children under the age of 5 years and <10 ml for children aged 5 years and above (European Medicines Agency, 2006). However, the more pleasant the formulation of the medicinal product tastes, the higher the dose volume which will be tolerated by the child. For example, 20 ml of an albendazole suspension are required to achieve a total dose of 400 mg. However, in large-scale drug administration programmes liquid formulations of albendazole, mebendazole or levamisole and pyrantel pamoate are rarely used. Albendazole, mebendazole and pyrantel pamoate are also available as chewable tablets. Although the use of chewable tablets is officially recommended for children aged above 6 years, a systematic review on the safety of chewable tablets for children in the US concluded that chewable tablets were a safe and well tolerated alternative to traditional paediatric formulations (Michele *et al*. 2002). Tragically, in 2007 in Ethiopia four children below the age of 3 years died from choking on chewable albendazole tablets during a deworming campaign (WHO, 2007*a*). Reasons for the widespread use of tablets in preventive chemotherapy campaigns are the higher cost of the suspension compared to tablets, difficulties of transport and storage due to the larger volume of the liquid formulation, stability issues and the difficulties in handling the liquid formulation. Hence, additional resources for implementation of liquid formulations would be required. Moreover, medication errors commonly occur with liquid formulations. It has been shown, for example that poor dose uniformity is achieved using spoons and dosing cups, hence dropper tubes or syringes are recommended (Breitkreutz and Boos, 2007).

Ivermectin

Ivermectin, the current drug of choice for strongyloidiasis, is marketed as 3 mg and 6 mg scored tablets. Ivermectin was approved for human use in 1988 and is widely used to control and eliminate filarial infections, usually in combination with albendazole (Fox, 2006; Taylor *et al*. 2010). Ivermectin is labelled for children weighting more than 15 kg, since the drug might cross the poorly-developed blood brain barrier in infants resulting in possible neurotoxic events (Fox, 2006). There is a need to study the safety of ivermectin in infants in greater detail. In addition, to our knowledge, liquid formulations are not available, hence preschool-aged children, are routinely treated with tablets, which is not the preferred dosage form for this age group (European Medicines Agency, 2006).

IMPLICATIONS FOR CONTROL

In the mid-1980s, a paradigm shift occurred in the global strategy against schistosomiasis and other major helminth infections; i.e. transmission control was gradually replaced by morbidity control. The advent of safe anthelminthic drugs that showed high efficacy at single oral doses, both in terms of cure rate and egg reduction rate, and hence morbidity



B

S. mansoni	Hookworm	Co-infection	No-infection
80%	80%	64%	4%
40%	40%	16%	36%
20%	20%	4%	64%

C

S. mansoni	Hookworm	Co-infection	No-infection
20%	20%	4%	64%
10%	10%	1%	81%
5%	5%	0.25%	90.25%

Fig. 6. Conceptual framework summarising the age-prevalence curve of schistosomiasis (a) and hypothetical scenarios of *S. mansoni*-hookworm co-infection in highly endemic (b) and low endemic (c) settings.

reduction, was at the root of this shift (WHO, 1985). Once the price of anthelminthics plummeted (e.g. terming out of patents or pharmaceutical companies providing anthelminthic drugs free of charge), repeated large-scale administration to at-risk populations became feasible. In May 2001, World Health Assembly (WHA) resolution 54·19 was endorsed, urging member states to regularly treat at least 75% of school-aged children at risk of schistosomiasis and soil-transmitted helminthiases with praziquantel and albendazole/mebendazole, respectively (WHO, 2002*b*). Major progress has been made over the past decade and, in 2006, the first African countries reached the 75% target of administering benzimidazoles to school-aged children at risk of soil-transmitted helminthiasis (WHO, 2008*b*, 2010*c*; Savioli *et al*. 2009).

With regard to schistosomiasis, however, only half a dozen countries in sub-Saharan Africa have (re-) established national schistosomiasis control programmes (Fenwick *et al*. 2009). Hence only a small fraction of school-aged children at-risk of morbidity due to schistosomiasis are regularly given praziquantel (Hotez *et al*. 2010; WHO, 2010*b*). In view of recent reports from different epidemiological settings documenting *S. haematobium* and *S. mansoni* single and even mixed species infections among preschool-aged children (Mafiana *et al*. 2003; Bosompem *et al*. 2004; Odogwu *et al*. 2006; Stothard and Gabrielli, 2007; Betson *et al*. 2010; Garba *et al*. 2010; Sousa-Figueiredo *et al*. 2010*b*; Stothard *et al*. 2011), the question has arisen whether preventive chemotherapy should be extended to this age group.

We welcome this discussion and an informal consultation held at WHO headquarters in Geneva in September 2010. We offer the following points for consideration, with Fig. 6 serving as a conceptual framework. Fig. 6A depicts a typical age-prevalence curve of schistosomiasis, clearly documenting that school-aged children are at highest risk of egg-patent infection and high infection intensity (Jordan and Webbe, 1969). Let us first focus on school-aged children (age: 5–19 years). The mean prevalence in this age group, based on detection of *S. mansoni* eggs in stool samples, is approximately 40%. Concurrently, the egg-patent prevalence of *S. mansoni* in preschool-aged children (age: 0–4 years) is approximately 10% or one-fourth of their older counterparts. Of note, immunodiagnostic markers, particularly antigens in urine, can be detected earlier than eggs in stool, and hence the respective prevalence based on antigens might be higher than that determined by egg patency (Stothard *et al*. 2011). Notwithstanding the shortcoming of egg patency at early age, let us assume a similar age-pattern of *S*. *mansoni* and hookworm infection.

Fig. 6B shows three hypothetical scenarios with school-aged children at risk of a *S*. *mansoni* infection, a hookworm infection, and a *S*. *mansoni*-hookworm co-infection. In an epidemiological setting where both infections are highly endemic and no control measures in place, i.e. 80% of school-aged children infected with either parasite, the likelihood of a co-infection is 64% under the assumption of random parasite distribution. We argue that such settings are rare to find in contemporary Africa. Suppose that control efforts have been launched and infection prevalences dropped by 50% for each parasite. The likelihood of a co-infection has now been reduced to 16%. Concurrently, 36% of the school-aged children are free of both *S*. *mansoni* and hookworm infection. Now, let us suppose that preventive chemotherapy is continued and the prevalence of either parasite infection further reduced to 20% each (Fig. 6C). The likelihood of a co-infection has further dropped to 4%, whereas two-third of the school-aged children are helminth-free. Next, let us focus on preschool-aged children where the prevalence of each parasite is one-fourth of the prevalence in their older counterparts. In the latter setting, less than 1% of preschool-aged children are co-infected, whereas more than 90% show no infection at all. Can preventive chemotherapy, using both praziquantel and a benzimidazole, which have yet to be properly tested in children below the age of 4 years, be justified in such a setting? There is a pressing need for additional studies, including risk-benefit analyses. For example, the risk of not treating the preschool-aged population in terms of potential morbidity should be determined and compared to the risk of administering two drugs that lack child-friendly formulations, and detailed information on efficacy, safety and PK in this age group. We conjecture that new research is urgently required to resolve these issues before extension of preventive chemotherapy to preschool-aged children.

CONCLUDING REMARKS AND RESEARCH NEEDS

Infectious diseases continue to be a leading cause of morbidity and mortality in children, particularly in low-income countries. As we have shown, there is a paucity of studies assessing the safety and efficacy of antiparasitic drugs in children. Moreover, PK investigations and studies in infants and adolescents with antiparasitics are a totally neglected research area. Though sometimes difficult to put into practice, PK studies should also assess the impact of the parasitic infection on the disposition kinetics of the drug, hence ideally be carried out in healthy and diseased children. For example, it has been demonstrated that PK parameters were altered in patients infected with the liver fluke *Opisthorchis viverrini* (Na Bangchang *et al*. 1993). Similarly, in many disease areas no progress has been made with tailored drug formulations for children. Hence, off-label use, empirical dose adaptations and formulation tampering are still the rule rather than the exception.

The forging of new alliances and public-private partnerships between academia, pharmaceutical companies and philanthropic organisations (e.g. Bill & Melinda Gates Foundation) hold promise to fill the current gaps for optimising current antiparasitic drugs and developing the next generation of antiparasitic drugs (Moran, 2005; Nwaka and Hudson, 2006; Keiser and Utzinger, 2007; Moran *et al*. 2009). The Medicines for Malaria Venture (MMV) and the Drugs for Neglected Disease *initiative* (DND*i*) can serve as useful role models (see Glossary).

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GLOSSARY

ADMET: Absorption, distribution, metabolism, excretion and toxicity

ATC: Anatomical Therapeutical Chemical classification system

BPCA: Best Pharmaceuticals for Children Act (2002)

DALY: Disability-adjusted life year is a composite measure to estimate the burden of disease or injury, expressed as the number of years lost due to ill-health, disability or premature death

DND*i*: Drugs for Neglected Diseases *initiative* is a collaborative, not-for-profit drug research and development organisation founded in 2003, based in Geneva, Switzerland that aims to improve the quality of life and the health of people suffering from neglected diseases by using an alternative model to develop drugs for these diseases and by ensuring equitable access to new and field-relevant health tools (http://www.dndi.org/)

FDA: US Food and Drug Administration (http://www.fda.gov)

FDAMA: FDA Modernization Act (1998)

IPT: Intermittent preventive therapy is a public health intervention aimed at treating and preventing malaria episodes in infants (IPT_i), children (IPT_c) and pregnant women (IPT_p). The intervention builds on two tested malaria control strategies, namely (1) to clear existing parasites (treatment effect seen in mass drug administrations) and (2) to prevent new infections (prophylaxis)

MMV: Medicines for Malaria Venture is a not-for-profit public-private partnership established in 1999, based in Geneva, Switzerland that aims to reduce the burden of malaria in disease-endemic countries by discovering, developing and facilitating delivery of new, effective and affordable anti-malarial drugs (http://www.mmv.org/)

Off-label: Use for this population/age class not mentioned in the label

Preventive (anthelminthic) chemotherapy: Use of (anthelminthic) drugs, either alone or in combination, as a public health tool against (helminth) infections

8.2 *Schistosoma mansoni* tetraspanning orphan receptor (SmTOR): a new vaccine candidate against schistosomiasis

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Schistosoma mansoni tetraspanning orphan receptor (SmTOR): a new vaccine candidate against schistosomiasis

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Summary

One approach to fight against schistosomiasis is to develop an efficient vaccine. *Schistosoma mansoni* tetraspanning orphan receptor (SmTOR) might be a vaccine candidate, as it is a tegument membrane protein expressed most highly in cercariae. In this study we characterized the recombinant first extracellular domain of SmTOR (rSmTORed1) as having the expected property to bind C2 of complement similarly to a smaller peptide of the same domain, and to produce specific and high-titre antibodies in BALB/c mice immunized using complete Freund's adjuvant/incomplete Freund's adjuvant (CFA/IFA). Immunization was protective against parasite infection, as demonstrated by a significant decrease in worm burden in immunized BALB/c mice *versus* the control groups over two independent trials [64 and 45% reduction for mean adult worm burden in immunized *versus* phosphate-bufferd saline (PBS) injected mice]. Interestingly, infection by itself did not lead to the generation of anti-rSmTORed1 antibodies, corresponding to the low frequency of specific anti-rSmTORed1 antibodies detected in the sera of patients infected with *S. mansoni* (2/20; 10%). These data suggest that, as opposed to the natural infection during which SmTOR induces antibodies only rarely, immunization with its smaller first extracellular domain might be more efficient.

Keywords: complement regulation, *Schistosoma mansoni* tetraspanning orphan receptor (SmTOR), schistosomiasis, tegument protein, vaccine candidate

Introduction

Schistosomes are parasitic helminths that are able to ensconce themselves in the human host for decades [1]. They were discovered in the mid-19th century [2], but must have infected their human hosts during thousands of years, as calcified eggs had already been discovered in mummies [3]. Their persistent existence over thousands of years might be one of the reasons why, during co-evolution with their human host, schistosomes developed into well-adapted parasites capable of escaping the host immune response and establishing themselves in such an unfriendly environment as the human venous system.

Today, an estimated number of 200 million people are infected with *Schistosoma* spp., with *S. mansoni*, *S. haematobium* and *S. japonicum* the most important species [4]. Despite many decades of research, praziquantel is the only chemotherapeutic drug available for treatment of schistosomiasis effective against all five schistosome species infecting humans [5]. Concern about the emergence of developing praziquantel resistance [6], the biphasic sensitivity of the parasite to the drug [7], with juvenile worm stages being insensitive to drug treatment [8], and the lack of protection against reinfection [9], are among the major disadvantages of a chemotherapeutic treatment of the infection. Consequently, the development of a schistosomiasis vaccine is highly desirable, although more than 10 years ago it was already stated to be a difficult but achievable goal [10]. This prediction proves true, in so far as no vaccine candidate is currently in the late stages of clinical development [11]. However, since 2009 the *S. mansoni* genome sequence has been fully available [12]. This remarkable achievement, together with a substantial amount of high-quality data generated by the various other '-omics' disciplines, pave the way for vaccine research against this and other schistosome species [13,14]. Some of the most interesting vaccine candidates are transmembrane proteins localized on the *S. mansoni* tegument, as they are seen immediately by the host immune system [15]. Proteins highly expressed in the early intramammalian stages of *S. mansoni*, such as schistosomula, are also considered to be favourable vaccine targets [16].

Schistosomes have to defend themselves against the host immune system at various stages in their life cycle [17–19]. The initial phase of invasion is characterized by evading innate immunity at the time of skin penetration and migration to small vessels. During this phase the host complement may attack the cercariae [20]. However, schistosomes express several complement regulators/inhibitors on their surface, which apparently block host complement [21]. We have recently described a new putative complement regulator in *S. mansoni*, termed SmTOR for tetraspanning orphan receptor [22]. This receptor is characterized by a 111 amino acid extracellular domain 1 (SmTORed1) containing the C-terminal H17 motif shown to bind C2 and interfere with its cleavage by C1s, thereby limiting the extent of the complement C3 convertase formation [23]. Its highest expression in cercariae and surface localization in *S. mansoni* early intramammalian stages would not only suggest a role for the receptor as a complement regulator at an early timepoint of infection, but also make it an interesting vaccine target.

In this work, we wanted to define whether recombinant SmTORed1 induces immune responses in mice and confers protection against infection. An additional question was whether or not humans infected with *S. mansoni* develop specific antibodies.

Materials and methods

Animals

Female C57BL/6 and female BALB/c mice ($n = 80$, age: 4 weeks, weight: ~14 g) used for the first round of immunization were purchased from Harlan Laboratories (Horst, the Netherlands). Female BALB/c mice ($n = 30$, age: 4 weeks, weight: ~14 g) used for the immunization challenge experiment were purchased from Charles River Laboratories (Sulzfeld, Germany). Animals were kept in groups of five (preliminary experiment) or 10 (immunization infection) in environmentally controlled conditions (temperature: 25°C; humidity: ~50%; 12-h light/dark cycle) and acclimatized for 1 week. They had free access to water and rodent diet. All experiments were approved by the ethical committees of the Swiss authorities at the Federal Veterinary Department (Bern, Switzerland) and the cantonal veterinary office Basel-Stadt (Switzerland) (permission number: 2346). They were conducted according to local guidelines (Verordnung Veterinäramt Basel-Stadt) and the Swiss animal protection law (TschG) at the Department of Biomedicine at the University Hospital Basel (first round of immunization) and at the Swiss Tropical and Public Health (TPH) Institute (Basel, Switzerland) (immunization challenge experiment).

Recombinant protein expression and purification

SmTORed1 ORF was cloned into the pET15b expression vector (Novagen, Merck Chemicals, Darmstadt, Germany) by the sticky-end polymerase chain reaction (PCR) method [24] using the *Nde*I and *Bam*HI restriction sites and pCR2·1-TOPO SmTOR [22] as template. Corresponding accession numbers for SmTOR deposited at the databases indicated are: SmTOR mRNA sequence, GenBank ID: JN560697; SmTOR full-length receptor sequence; and UniProt ID: C4QM85.

The primer pairs used were, for PCR 1, 5′-TATGCCGA GACTTATTCCAGAGGATAAT-3′ (forward 1) and 5′-GAT CCTTAGTAAGGACTGAAATGCTTTAT-3′ (reverse 1); and for PCR 2, 5′-TGCCGAGACTTATTCCAGAGGATAAT-3′ (forward 2) and 5′-CGATCCTAGTAAGGACTGAAATGC TTTAT-3′ (reverse 2). Sticky-end PCR was performed as follows: two different PCR reactions were performed, reaction products were cleaned up separately by agarose gel electrophoresis and purification of the bands at the expected molecular weight [345 base pairs (bp)] was performed using the QIAquick gel extraction kit (Qiagen, Hombrechtikon, Switzerland). The extracted DNA was then mixed in equimolar ratio (1:1), denatured at 95°C for 5 min and then annealed on ice. pET15b vector was prepared by double digestion with *Nde*I and *Bam*HI (NEB, Frankfurt, Germany) and purified as described above. Ligation was performed using Quick Ligase (NEB) and the mixture was transformed into TOP 10 bacteria (Invitrogen, Carlsbad, CA, USA) and grown on Luria broth (LB) agar plates containing 100 μg/ml ampicillin (Sigma, St Louis, MO, USA). Clones were analysed by restriction enzyme digestion with *Sal*I (NEB) cutting at position 244 of the insert sequence. Positive clones were sequenced and used for transformation into a bacterial expression strain.

SmTORed1pET15b was transformed into BL21 (DE3) bacteria for protein overexpression. Batch cultures were grown in autoinduction media, as described by Studier [25]. Pre-cultures were grown in MDAG non-inducing medium and main cultures were grown in MDA-5052 autoinducing medium, both supplemented with 100 μg/ml carbenicillin (Sigma). Single clones of SmTORed1pET15b (rSmTORed1 purification) or pET15b (mock transfection, purification of control fraction) transformants were picked and pre-cultures were grown overnight at room temperature (RT) with shaking at 220 rpm and diluted subsequently at 1:50 in MDA-5052 medium for protein expression. Fifty ml main cultures in 250 ml Erlenmeyer flasks were shaken at 250 rpm for 18 h at RT. Bacteria were harvested by centrifugation and pellets frozen at −20°C until required.

Recombinant protein was purified from the insoluble cytoplasmic fraction (inclusion bodies) of SmTORed1 pET15b transformed BL21 (DE3) bacteria. Bacteria were lysed using BugBuster bacterial cell lysis detergent (Novagen) supplemented with ethylenediamine tetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), and inclusion bodies were purified by repeated centrifugation and washing steps as described in the pET system manual (Novagen). The final inclusion body pellet was resuspended in 10 ml denaturing buffer (buffer A) containing 10 mM imidazole (50 mM NaH_2PO_4, 500 mM NaCl, 6 M guanidine hydrochloride, pH 8 + 10 mM imidazole). The recombinant protein was then purified by metal affinity chromatography using ÄKTAprime plus system (GE Healthcare Biosciences, Piscataway, NJ, USA). A 5-ml Ni-NTA Superflow Cartridge was equilibrated with buffer A and 5 ml of solubilized sample loaded onto the column (one cleaned-up batch corresponded to 25 ml original bacterial culture). The column was then washed with five bed volumes of buffer A and then eluted with a linear gradient (gradient volume 100 ml) of buffer A to 100% buffer B (50 mM NaH_2PO_4, 500 mM NaCl, 6 M guanidine hydrochloride, pH 8 + 250 mM imidazole). Fractions containing the main protein peak were pooled and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (15%). Purified histidine (His)-tagged rSmTORed1 was refolded by stepwise dialysis. First, the sample (in a volume of 20–25 ml buffer A/buffer B) was dialysed into refolding buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 400 mM L-arginine) at 4°C overnight using a dialysis membrane of 3500 molecular weight cut-off (Spectrum Laboratories, Breda, The Netherlands). Two additional dialysis steps against 1 × PBS were then performed for 3 h at 4°C each. The purity and concentration of the sample were then evaluated analysing an aliquot by 15% SDS-PAGE and bands visualized by Coomassie staining (Instant Blue; Expedeon, Harson, UK). Molecular weight markers used were Precision Plus Protein standards (Bio-Rad, Munich, Germany) and BenchMark ladder (Invitrogen). Protein concentration in the sample was determined using DC protein assay (Bio-Rad). The Coomassie-stained protein band at the expected molecular weight was excised and analysed by in-gel tryptic digestion and liquid chromatography–mass spectrometry (LC-MS/MS) analysis [26]. An inclusion body control fraction was prepared in *Escherichia coli* by transformation with pET15b plasmid analogous to the expression and purification of the rSmTORed1 protein.

Characterization of SmTORed1 by Western blot and enzyme-linked immunosorbent assay (ELISA)

Western blot using anti-ed1 antibody AbyD04644·1 (Serotec) was performed as published previously [22]. Protein samples were run on a standard 15% acrylamide/bisacrylamide gel (Bio-Rad) and transferred to a nitrocellulose membrane. The membrane was blocked in phosphate-buffered saline-0·05% Tween 20 (PBST)/5% milk followed by incubations with primary (AbyD04644·1, 1:1000) and secondary antibody [goat F(ab′)2 anti-human immunoglobulin (Ig)G-horseradish peroxidase (HRP)] (1:3000; Serotec, Martinsried, Germany) in PBST 1% milk, each for 1 h at RT. The blot was developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

For enzyme-linked immunosorbent assay (ELISA) measurements, 96-well plates (Maxisorp, Nunc, Rosklide, Denmark) were coated overnight at 4°C with rSmTORed1 (500 nmol/well) in 0·1 M bicarbonate buffer, pH 9·6. Plates were then blocked with 5% bovine serum albumin (BSA) in 1 × PBST for 1 h at RT. After blocking, AbDy04644·1 (2·51 mg/ml stock) diluted in PBST was used at dilutions ranging from 1:1000 to 1:10 000 to detect rSmTORed1 by ELISA. Anti-green fluorescent protein (GFP) AbDy04652·1 (1·14 mg/ml stock) diluted 1:4545 and 1:454 was used as negative control, corresponding to the highest (1:10 000) and lowest dilutions (1:1000) of AbDy04644·1, respectively. The plates were incubated for 45 min at RT with the primary antibody and were then washed five times with PBST. Secondary antibody [goat F(ab′)2 anti-human IgG-HRP; Serotec] diluted 1:10 000 was added for another 30 min at RT, and after washing the ELISA was developed by incubation with a 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate (BD Pharmingen, Allschwil, Switzerland). The absorbance was measured at 450 nm. For testing C2 binding to rSmTORed1 by ELISA, plates were coated and blocked as described above. Then, purified complement C2 (CompTech, Tyler, TX, USA) was diluted in 20 mM Tris buffer + 1 mM $MgCl_2$ + 1 mM $CaCl_2$ (amounts as indicated in figures) and incubated for 1 h at RT. Plates were washed and incubated with anti-C2 antibody (Calbiochem, Läufelfingen, Switzerland) diluted 1:5000 in PBST for 45 min at RT. After additional washes, plates were incubated with donkey anti-goat HRP, 1:10 000 in 1 × PBS for 30 min at RT. Finally, plates were washed and developed as described previously.

Detection of anti-rSmTORed1 antibodies in human sera by ELISA

Samples of anonymous patient sera were provided from an archived bank of samples collected (at the Swiss TPH, Basel) from known positive *S. mansoni*-infected individuals. Sera had been tested positively for antibodies against soluble worm antigen (SWA) and soluble egg antigen (SEA) and by immunofluorescence antibody tests (IFAT). Serum samples from uninfected individuals living in Switzerland were obtained from the Blutspendezentrum, Basel.

Nunc Maxisorp 96-well plates were coated with 1 μmol rSmTORed1 per well diluted in 0·1 M bicarbonate buffer, pH 9·6 overnight at 4°C. Plates were washed three times with PBST, and serum samples diluted 1:500 in PBST were added for 45 min, 37°C. After incubation with sera, plates were washed five times with PBST and biotinylated secondary antibody diluted 1:10 000 was added to each well [goat F(ab′)2 anti human IgG; Biosource] for 1 h at RT. This was followed by an additional five washes and 30 min incubation with streptavidin–HRP (Pierce; 1:10 000 in 1 × PBS) at RT. After washing, ELISA was developed using TMB peroxidase substrate and the absorbance at 450 nm was measured.

Cloning of HaloTag fusion constructs

All N-terminal HaloTag fusion constructs were cloned with the primers listed in Table S1 into the bacterial T7 promoter based expression vector pFN18A HaloTag T7® Flexi® Vector (Promega, Madison, WI, USA). The barnase-positive selection cassette was removed by digestion of pFN18A with *Pvu*I and *Pme*I and replaced by Halofwd and Halorev annealed oligos, as described previously [27]. The construct pFN18A Halo expressing HaloTag only was used as negative control. Halo-SmTORed1 (111 aa) and Halo-C4beta (C4beta: 26 aa corresponding to C4b$^{206–232}$ peptide stretch) were amplified from pCR2·1-TOPO SmTOR [22] for the SmTORed1 constructs or Hep2G (human hepatoma cell line) cDNA for the C4beta chain peptide. Cloning was performed with the sticky-end PCR method [24] using iProof high-fidelity DNA polymerase (Bio-Rad) and the inserts thus generated were ligated into *Pvu*I/*Pme*I digested pFN18A vector with Quick Ligase (New England Biolabs, Frankfurt, Germany). Plasmids were propagated in TOP10 *E. coli* (Invitrogen) and open reading frames verified by sequencing.

Overexpression and purification of Halo-tagged peptides

Plasmids (pFN18A Halo, pFN18A Haloed1 and pFN18A Halo C4 beta) were transformed into chemically competent BL21 (Sigma). Single clones were picked from LB/ampicillin plates and were grown overnight at 37°C (at 220 rpm) in 2 ml LB medium supplemented with 100 μg/ml ampicillin (Sigma). Pre-cultures were diluted 10-fold in LB/ampicillin and when grown to an optical density (OD)$_{600}$ of >0·6 induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Aliquots of the cultures were screened for expression of target gene 3–5 h after induction by SDS-PAGE and Coomassie staining. Total cell protein fractions were prepared using PopCulture reagent (Novagen) according to the protocol in the pET system manual (Novagen). Theoretical molecular weights of the fusion constructs were calculated as 35·4 kDa (Halo), 38·5 kDa (Halo C4 beta) and 48·2 kDa (Haloed1).

In order to purify Halo-tagged constructs, bacteria cultures (V = 20 ml) were pelleted, resuspended in BugBuster mix (Novagen) and soluble cytoplasmic fractions were prepared according to the manufacturer's protocol. Protein samples were then prepared for coupling onto magnetic beads (HaloLink, G9311; Promega). Buffer exchange into 1 × PBS was accomplished by using 3000 MWCO Amicon Ultra Centrifugal Filter Devices (Millipore, Volketswil, Switzerland). Halo protein concentrations were estimated in comparison with marker protein bands (BenchMark Protein Ladder; Invitrogen; each protein present at a concentration of approximately 0·1 mg/ml). For the coupling reaction, samples were diluted at the appropriate concentrations in 100 mM TrisHCl, pH 7·5, 150 mM NaCl, 0·01% NP40. HaloTag fusion proteins were immobilized on magnetic beads as described in the instruction manual (G9311; Promega). Magnetic beads were incubated with the HaloTag fusion proteins in excess in order to ensure that equal amounts of protein, i.e. beads, were used in the assay. This was the case because the covalent bond formation between HaloTag and chloralkane linker is specific and highly irreversible [28]. After coupling reactions, beads were washed extensively in PBS and used for competition ELISA or peptide purification. An amount of 40 μg Halo-tagged protein was coupled onto 20-μl beads in gel slurry that was then used in the competition ELISA.

Competition ELISA assay to measure specific anti-rSmTORed1 antibodies in human sera

For competition ELISA, 96-well plates were coated as described above, but with 500 nmol rSmTORed1 per well. Anti-rSmTORed1 antibody measurement in human sera was performed exactly as described above, but sera were first incubated with Halo-tagged fusion constructs, described as follows. Twenty μl washed beads with immobilized Halo or Haloed1 were resuspended in 250 μl PBST. One ml of serum sample (resulting in a serum dilution of 1:250) was added and the mix incubated on a wheel for 20 min at RT. As a control, 1 μl of serum sample was diluted in 250 μl PBST and incubated in parallel without beads. The coated ELISA plate was washed and 50 ml of PBST added per well. Magnetic beads were removed after incubation with the diluted serum and 50 μl of depleted serum (or control sample) was added per well (experiments were performed in triplicate), resulting in a final serum dilution of 1:500. Plates were incubated at 37°C for 45 min and the protocol proceeded as described above.

Vaccination of mice with rSmTORed1 – immunization and immunization challenge schedules

In the first round of immunization, four different mouse strain/adjuvant combinations were tested. As adjuvant, either complete Freund's adjuvant (CFA: first immunization and incomplete Freund's adjuvant (ICF: boosting) (experimental groups 1 and 3) or muramyl dipeptide (MDP: first immunization and boosting) (experimental groups 2 and 4) were used. The four experimental groups were: group 1: BALB/c mice injected with rSmTOR or control injections (see below) using CFA/IFA ($n = 20$); group 2: BALB/c mice injected with rSmTORed1 or control injections using MDP ($n = 20$); group 3: C57BL/6 mice injected with rSmTORed1 or control injections using CFA/IFA ($n = 20$); and group 4: C57BL/6 mice injected with rSmTORed1 or control injections using MDP ($n = 20$). Within these four groups, there were three subgroups consisting of 10 animals immunized with rSmTORed1 in PBS plus adjuvant (subgroup 1, immunized), five animals injected with inclusion bodies in PBS plus adjuvant (subgroup 2, first control group) and five animals injected with 1 × PBS plus adjuvant (subgroup 3, second control group). Immunization took place on day 0 followed by boosts at days 21 and 42. Ten µg of purified rSmTORed1 in PBS/adjuvant, inclusion bodies in PBS/adjuvant or PBS/adjuvant alone were used per injection at the days indicated. Mice were injected subcutaneously in the neck fold. Bleeding to monitor antibody response was performed at days −7, 14, 35 and 63 via the tail vein. Mice were killed at day 63 and spleens were dissected for generation of spleen cell cultures.

Immunization challenge experiments were performed using BALB/c mice (Charles River, Sulzfeld, Germany) in combination with CFA/IFA only. Groups consisted of mice injected with rSmTORed1 ($n = 10$, trials 1 and 2), inclusion bodies ($n = 10$, trial 1) or PBS ($n = 10$, trials 1 and 2) mixed with adjuvant, as tested in the preliminary round. Mice were infected at day 55 (13 days after the second boost). Cercariae of *S. mansoni* (Liberian strain) were harvested from infected intermediate host snails (*Biomphalaria glabrata*) maintained at laboratories at the Swiss TPH after exposure to light for 3 h. Mice were infected subcutaneously with 150 *S. mansoni* cercariae. At day 105 (7 weeks post-infection) mice were killed and parasite status was assessed by blinded evaluation of the adult worm burden. In trial 2, two mice of the control group (PBS injected) were not infected and were not included in the analysis.

Worm burden and liver egg burden analysis

Worms were dissected from the mesenteric veins of the mice. Protection values were calculated as described elsewhere [29]. Livers were then weighed and eggs and worms were counted microscopically in pressed livers using a counting grid. The average number of eggs per square was determined by counting eggs in three squares/liver and total liver area, i.e. number of grids per liver, was determined. Number of eggs per gram liver (epg) was calculated by dividing total number of eggs per liver by the liver weight. The spleens were removed in order to generate spleen cell cultures. Serum samples to assess antibody response were taken at days −7, 14, 35 as in the preliminary round (pre-challenge) and between days 105 and 107 (post-challenge).

Mouse serology

Specific antibody titres against rSmTORed1 were measured in individual mouse sera using an ELISA assay. For this, 96-well flat-bottomed microtitre plates (Nunc) were coated overnight at 4°C with 500 nmol rSmTORed1 per well in 100 µl 0·1 M carbonate bicarbonate buffer, pH 6·8. The plates were then blocked in 1 × PBST/5% BSA for 1 h, 37°C. Plates were washed five times with PBST between the different incubation steps. Serum samples were diluted in PBST at the appropriate dilution determined by serial dilutions for individual samples of each of the possible mouse strain adjuvant combinations and the different detection antibodies (described below), and 100 µl were then added per well. Plates were incubated for 1 h at RT, and after washing the biotinylated primary antibody diluted in PBST was then added for another hour at RT. Bound antibody was detected with streptavidin–HRP (Pierce) diluted 1:10 000 in PBS (30 min, RT) and the ELISA was developed by incubation with a TMB peroxidase substrate (BD Pharmingen). The absorbance was measured at 450 nm.

Biotinylated detection antibodies used were: goat anti-mouse IgG+IgM+IgA (ab6005; AbCam, Cambridge, UK) diluted 1:10 000, goat anti-mouse IgG (ab5868; AbCam) diluted 1:10 000, goat anti-mouse IgM (ab5929; AbCam) diluted 1:20 000, goat anti-mouse IgE (no. 1110-08; SouthernBiotech, Birmingham, AL, USA) diluted 1:5000, goat anti-mouse IgA (no. 1040-08; SouthernBiotech) diluted 1:10 000, rat anti-mouse IgG1 (no. 406604; Biolegend, San Diego, CA, USA) and rat anti-mouse IgG2a (no. 407104; Biolegend) both diluted 1:500. Serial dilutions to determine the working serum dilution were performed for the individual mouse strain adjuvant combinations as shown in Supplementary Fig. S3. For Ig serology (IgG + IgM + IgA determination), sera of BALB/c CFA/IFA groups were diluted 1:25 600; C57BL/6 CFA/IFA sera were diluted 1:6400 and sera of MDP vaccinated mice were diluted 1:3200 for BALB/c and C57BL/6 mice. For IgG, IgM, IgE, IgA, IgG1 and IgG2a determination in BALB/c CFA/IFA-vaccinated mice, sera were diluted 1:12 800 for IgG measurement and 1:1600 for all other antibody isotype levels measured, as determined by end-point titrations as well (data not shown).

Spleen cell cultures

Freshly removed spleen from infected and uninfected mice was cut into small pieces and the suspension pressed through a 70-µm nylon mesh (BD Falcon, BD Biosciences, Allschwil, Switzerland; no. 352350) into a 15-ml Falcon tube containing RPMI-1640 medium supplemented with 5% fetal calf serum (FCS) (RPMI/FCS). Cells were pelleted, resuspended in 5 ml lysis buffer (0·15 M NH_4Cl, 15 mM $KHCO_3$, 0·1 mM Na_2EDTA, pH 7·4) and incubated for 5 min at RT. Cell lysis was stopped by adding 10 ml RPMI/FCS and centrifuged for 10 min, 200 ***g*** at 4°C. Cells were resuspended in complete medium (RPMI/FCS + 2 mM L-glutamine, 50 μM beta-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin sulphate + 250 ng/ml amphotericin B + 30 μg/ml polymyxin B) and cell number adjusted to 1×10^7 cells per ml. Cells were cultured at 1×10^6 cells/ml in 200 ml in a 96-well tissue culture plate, either stimulated with 10 μg/ml rSmTORed1 or 5 μg/ml concanavalin A. Cell culture supernatants were collected after 48 h of stimulation for interleukin (IL)-4 analysis and after 72 h for IL-10 and interferon (IFN)-γ. Mouse cytokine ELISA measurements were performed using ELISA sets (BD OptEIA^{TM}, BD Biosciences, Allschwil, Siwtzerland). Stimulation with concanavalin A was performed to ensure the responsiveness of the splenocytes to stimulation and was positive for all the cultures and cytokines measured (mean values 8500 pg/ml and 850 pg/ml for IFN-γ and IL-10 for samples shown in Supplementary Fig. S6a and 150 pg/ml for IL-4, not detectable in rSmTORed1 stimulated cultures). The differences in the level of responses between the two sets of experiments (immunization alone, Supplementary Fig. S6a; immunization + challenge, Supplementary Fig. S6b) were due to cytokine kits as indicted by internal controls used in both series of tests.

Immunofluorescence staining and confocal microscopy

S. mansoni schistosomula were prepared as described elsewhere [30]. After incubation in medium for the indicated number of hours and washing in 1 × PBS, schistosomula were either embedded directly in octreotide (OCT) or used for staining of the whole parasite. Nine-μm OCT sections of 3-h schistosomula were fixed in ice-cold methanol for 10 min, blocked in PBST/5% BSA and incubated with anti-rSmTORed1 sera (pooled sera from 10 BALB/c mice immunized with rSmTORed1, bleeding at day 63) or pre-immune serum (pooled sera from BALB/c mice immunized with rSmTORed1 but bled before first injection, bleeding day −7) diluted 1:25 in PBST/3% BSA for 2·5 h at RT. After four washes, slides were incubated for 1 h with Alexa Fluor® 488 rabbit anti-mouse IgG (H + L) (A11059; Molecular Probes, Eugene, OR, USA) diluted 1:150 in PBST/3% BSA and washed again before mounting. Whole parasites (24-h schistosomula) were fixed 1 h in 4% paraformaldehyde/PBS on ice and after washing five times with PBS were blocked and stained as described for OCT sections above. All slides were mounted with Vectashield fluorescence mounting medium (Vector Laboratories, Peterborough, UK) and examined using the LSM 510 META confocal laser scanning microscopy system (Carl Zeiss, Feldbach, Switzerland) with a Zeiss Plan Neofluar 63×/1·25 numeric aperture oil (1/0·17) objective.

Statistical analyses

Student's *t*-test was used to compare Ig amounts in mouse sera at two different time-points, and to compare experimental and control group (immunization challenge trial 2) on worm burden and liver egg burden. Statistical differences between antibody amounts of Ig at day 35 and differences in worm burden between three different groups (trial 1) were determined by one-way analysis of variance (ANOVA) (Kruskal–Wallis) followed by Dunn's multiple comparison test. Linear correlation between female worm burden and epg liver was evaluated using Spearman's rank correlation analysis.

Results

First, the recombinant SmTORed1 was prepared and characterized, then used in mice to define immunogenicity and protection against infection. Finally, we investigated human sera for the presence of specific anti-SmTORed1 antibodies.

Recombinant SmTORed1, purification and molecular characterization

In order to test the immunogenicity of the SmTOR extracellular domain 1, we produced it as an N-terminally His-tagged fusion protein rSmTORed1 containing only a short linker sequence (Fig. 1a). SmTORed1 ORF was cloned into a pET15b vector where its expression was manageable under the control of the lac repressor [31]. An aliquot of the fast protein liquid chromatography (FPLC)-purified recombinant SmTORed1 dissolved in PBS was analysed by gel electrophoresis before and after the final centrifugation step and a protein band of the calculated molecular weight of 14·7 kDa was detected (Fig. 1b). The protein band was sequenced by mass spectrometry and fragments belonging to recombinant SmTORed1 were identified (Fig. 1a). The protein concentration of total compared to soluble rSmTORed1 was 145 ng/μl and 132 ng/μl, indicating that only a small portion of rSmTORed1 was not soluble under optimized conditions for refolding in a large volume of buffer. Pooled protein fractions analysed before refolding into 1 × PBS showed an additional protein band at an approximate molecular weight of 30 kDa (Fig. 1c). No other bands were detected by Coomassie staining in this or other similarly concentrated samples of purified rSmTORed1. Recombinant SmTORed1, as well as the 30 kDa protein band at the size of a potential peptide dimer, were detected by Western blot using monoclonal anti-ed1 antibody directed against the 27 C-terminal amino acids of rSmTORed1 (Fig. 1c). The same monoclonal antibody recognized plate-bound rSmTORed1 (data not shown), and rSmTORed1 was also shown to bind C2, as expected [23,32] (Fig. 1d).

It has been shown previously that complement C2 binds to the β chain of C4, specifically to a peptide sequence in

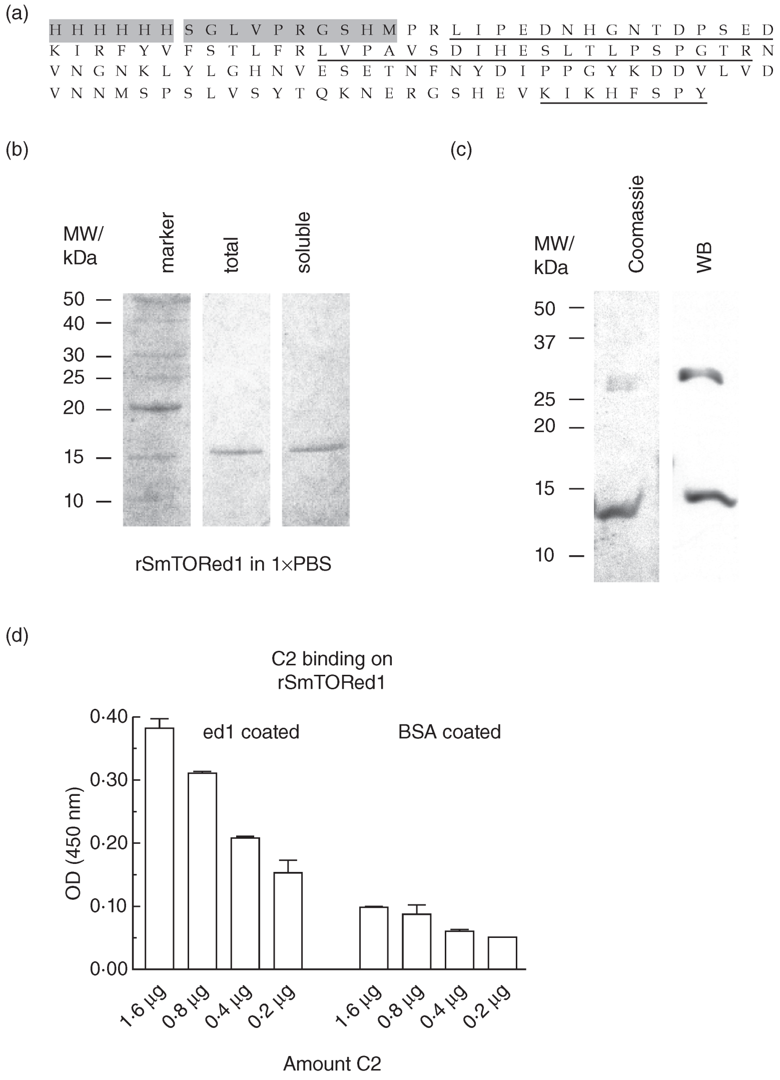

Fig. 1. Production of histidine (His)-tagged *Schistosoma mansoni* tetraspanning orphan receptor extracellular domain 1 (SmTORed1) in *Escherichia coli* and molecular characterization of purified peptide by mass spectrometry, Western blotting and enzyme-linked immunosorbent assay (ELISA). (a) Recombinant first extracellular domain of rSmTORed1 peptide sequence with the 6 × His-tag and linker sequence (10 aa) originating from the pET15b vector shaded in grey. Fragments identified by mass spectrometry are underlined. (b) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified rSmTORed1 after Ni^{2+} chromatography (pooled eluates) and refolding into 1 × phosphate-buffered saline (PBS). Total amount of peptide in buffer was analysed before (total, lane 2) and after removal of residual precipitate by centrifugation (soluble, lane 3). Four μl of sample was loaded per lane. Lane 1 (marker): benchmark protein ladder. Protein bands are visualized by Coomassie stain. (c) Pooled fractions of fast protein liquid chromatography (FPLC)-purified peptide analysed before dialysis into 1 × PBS; 20 μl sample was loaded per lane. Peptides detected by Coomassie blue (left) and by Western blot using monoclonal anti-ed1 antibody AbDy04644·1 (right). (d) C2 binding on a rSmTORed1 coated ELISA plate, binding in 20 mM Tris buffer + 1 mM $MgCl_2$ + 1 mM $CaCl_2$. ELISA Results are expressed as means of individual measurements. Error bars indicate standard deviation (s.d.) of the means.

C4β that is homologous to the SmTORed1 peptide [23]. Thus antibodies (human and mouse) that are specific for SmTORed1 might cross-react with C4. We used SmTORed1 peptide and the homologous sequence on the C4β chain peptide aligning with it (termed C4beta) produced as N-terminal Halo-tagged bacterial fusion proteins and cut by digestion with tobacco etch virus (TEV) protease along with rSmTORed1 to demonstrate that there was no cross-reactivity detected by ELISA (Supplementary Figs S1, S2).

Evaluation of the humoral immune response in BALB/c and C57BL/6 mice immunized with rSmTORed1

To define the immunogenicity of rSmTORed1, we immunized two different mouse strains with rSmTORed1 formulated with CFA/IFA or MDP as adjuvant. After initial immunization, mice were boosted twice at 3-week intervals. After two injections with antigen and the CFA/IFA as adjuvant, BALB/c mice showed a significant increase in Ig levels detectable at high dilution compared to levels in pre-immune sera (Fig. 2a) whereas no antibodies were produced in the inclusion body and PBS-injected mice at the same time-point (Fig. 2a). BALB/c mice responded to the second booster injection with peptide and IFA with sustained production of Ig to rSmTORed1 (data not shown). In fact, dilutions set for Ig analysis had been determined by end-point titrations (Supplementary Fig. S3). Sera were diluted 1:25 600 for Ig analysis of CFA/IFA-immunized BALB/c and specific antibodies in these mice were detected at a dilution as high as 1:1 638 400 compared to pre-immune sera (Supplementary Fig. S3a). CFA/IFA-vaccinated C57BL/6 mice and both strains of MDP-vaccinated mice generated low (Supplementary Fig. S3b) to very low (Supplementary Fig. S3c,d) amounts of

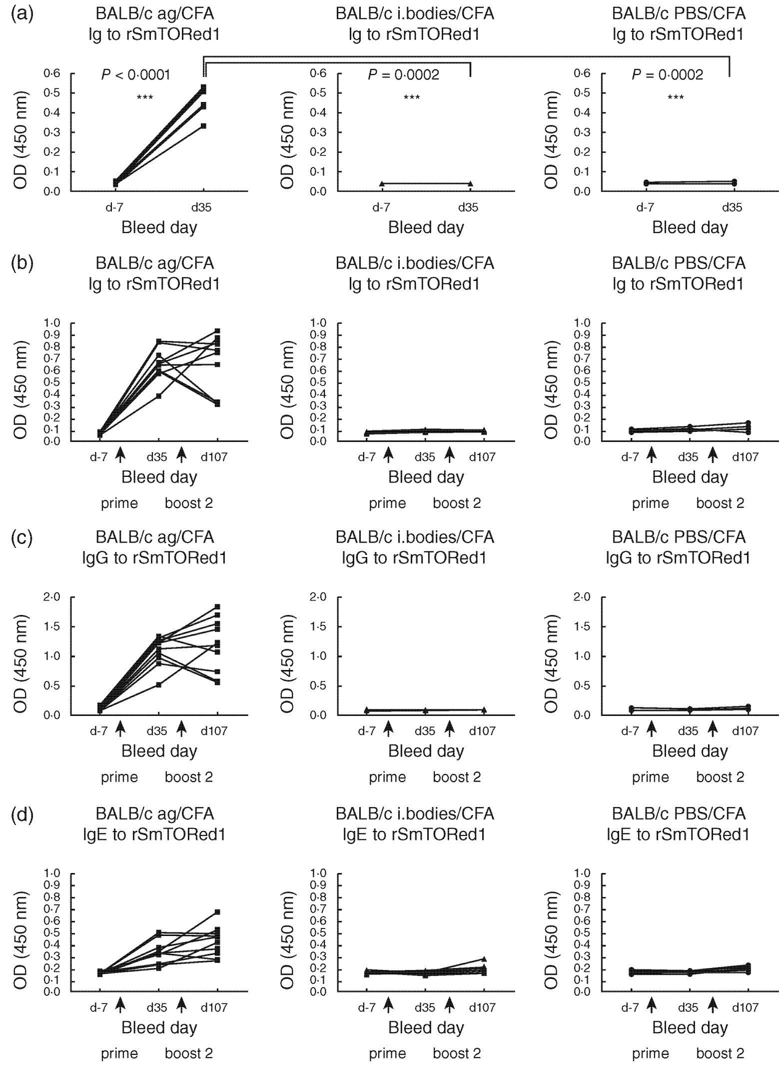

Fig. 2. Recombinant first extracellular domain of *Schistosoma mansoni* tetraspanning orphan receptor (rSmTORed1) complete Freund's adjuvant/incomplete Freund's adjuvant (CFA/IFA)-induced antibody response in BALB/c mice (a) after immunization only, and (b–d) after immunization followed by a challenge with *S. mansoni* infection. (a) Total immunoglobulin (Ig) amounts in sera of individual BALB/c mice immunized with rSmTORed1/CFA/IFA (antigen, ag; $n = 10$) and the control groups injected with inclusion bodies/CFA/IFA (i. bodies; $n = 5$) or phosphate-buffered saline (PBS)/CFA/IFA (PBS; $n = 5$) measured in sera pre-immunization (day −7) and after the first boost (day 35). Sera for the enzyme-linked immunosorbent assay (ELISA) assay were diluted 1:25 600 as determined by end-point titrations. Statistical analyses used were: Student's *t*-test to compare titre at days −7 and 35 and one-way analysis of variance (ANOVA) to compare titres at day 35; n.s.: statistically not significant; statistically significant *($P < 0·05$), **($P < 0·01$), ***($P < 0·001$). (b–d) Total Ig (b), IgG (c) and IgE (d) amounts in sera of individual BALB/c mice immunized with rSmTORed1/CFA/IFA (antigen, ag; $n = 10$) and the control groups injected with inclusion bodies/CFA/IFA (i. bodies; $n = 10$) or PBS/CFA/IFA (PBS; $n = 10$) all infected with *S. mansoni* cercariae at day 55. Sera were sampled at days −7, 35 and 107 and measured at serum dilutions of 1:25 600 (total Ig), 1:12 800 (IgG) and 1:1600 (IgE). Arrows indicate injections at days 0 (prime), 21 (boost 1) and 42 (boost 2).

anti-rSmTORed1 Ig and sera were therefore tested at low dilutions. Indeed, immunization of C57BL/6 mice with rSmTORed1 in CFA/IFA and BALB/c mice with rSmTORed1 in MDP also resulted in a significant but small increase of Ig levels measured at low dilution (Supplementary Fig. S4a,b). This increase was not uniformly detectable in all animals of the immunized groups and not significant overall compared to the control groups. Finally, no significant Ig levels were detected in C57BL/6 mice immunized with rSmTORed1 in MDP (Supplementary Fig. S4c). No Ig bound on the immobilized inclusion body fraction, and mouse Ig to rSmTORed1 did not cross-react with the TEV cut rC4beta peptide sequence (Supplementary Fig. S2). Control animals, both mice receiving inclusion bodies together with adjuvant and mice receiving adjuvant mixed with buffer only, did not show an increase in anti-rSmTORed1-specific immune response in any set-up.

Vaccination of BALB/c mice and challenge with *S. mansoni* cercariae

We chose to perform an immunization challenge experiment using BALB/c mice in combination with CFA/IFA to determine whether or not the immune response generated in these mice upon vaccination with rSmTORed1 would have a protective effect against *S. mansoni* infection. Antibodies generated against rSmTORed1 persisted during the entire duration of the experiment and levels were not altered significantly in most of the animals after infection with *S. mansoni* (Fig. 2b). Immunized mice produced antigen-specific IgG and IgE (Fig. 2c,d), whereas specific anti-rSmTORed1 IgM and IgA levels were not detected (Supplementary Fig. S5).

Cytokine secretion by spleen cell cultures induced by stimulation with peptide were analysed to assess the

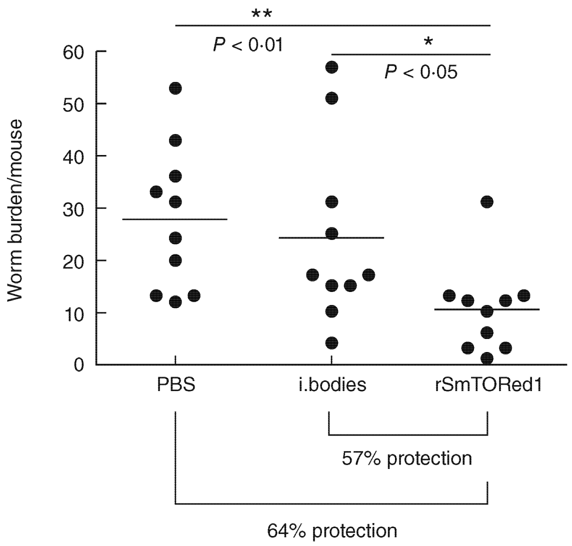

Fig. 3. Scattergram of total worm burden of BALB/c mice (trial 1) immunized with complete Freund's adjuvant/incomplete Freund's adjuvant (CFA/IFA) adjuvanted recombinant first extracellular domain of *Schistosoma mansoni* tetraspanning orphan receptor (rSmTORed1), inclusion bodies (i. bodies) or phosphate-buffered saline (PBS) and challenged with *S. mansoni* cercariae. The number of animals used per group was *n* = 10. Mean worm burden ± standard deviation (s.d.) for the different groups were 28·8 ± 14·6 (PBS), 24·2 ± 17·4 (inclusion bodies) and 10·4 ± 8·6 (rSmTORed1). Statistical analyses were performed using one-way analysis of variance (ANOVA) (Kruskis–Wallis) followed by Dunn's multiple comparison test. Statistically significant *(*P* < 0·05), **(*P* < 0·01).

T helper cell polarization induced by the immunization of BALB/c mice with rSmTORed1 in CFA/IFA. Spleen cell cultures that were performed 14 days after the second booster with rSmTORed1 produced significant amounts of IFN-γ and IL-10, but no IL-4 when stimulated with the antigen, compared to the spleen cell cultures originating from control animals (Supplementary Fig. S6a). The same cytokine profile was detected in spleen cell cultures of immunized animals after challenge with infection (Supplementary Fig. S6b). Control experiments using concanavalin A confirmed that all spleen cell cultures were capable of producing all three cytokines tested (not shown).

The status of infection in animals immunized with rSmTORed1 and challenged by injection of 150 *S. mansoni* cercariae was evaluated by analysing total worm burden (7 weeks post-infection). We found that mice immunized with rSmTORed1 showed a significant reduction in worm burden when compared to both control groups (Fig. 3). Vaccination resulted in 57% reduction of worm burden when compared to the mice injected with the inclusion body fraction and a reduction of 64% when compared to the PBS control mice. In a second independent trial, vaccination with rSmTORed1 resulted in 45% reduction of mean adult worm burden and a 50% reduction in mean liver egg burdens when compared to the PBS-injected mice (Table 1). There was a strong correlation between number of female worms and number of eggs per gram liver analysing all values (*n* = 18) of immunized and control groups (Spearman's *r* = 0·8589, *P* < 0·0001).

Table 1. Parasitological data of BALB/c mice vaccinated with recombinant first extracellular domain of *Schistosoma mansoni* tetraspanning orphan receptor (rSmTORed1), inclusion bodies (i. bodies) or phosphate-buffered saline (PBS) all adjuvanted with complete Freund's adjuvant/incomplete Freund's adjuvant (CFA/IFA).

	Adult worms, range	Mean worm burden ± s.d. % reduction, *P*-value			Total worm burden Mean ± s.d. % reduction, *P*-value	Number of eggs per gram liver tissue; epg × 10^3 Mean ± s.d. % reduction, *P*-value
		Male	Female	Pairs		
Trial 1						
Control (PBS) *n* = 10	12–53	16·5 ± 8·3	12·3 ± 6·7	8·7 ± 6	28·8 ± 14·6	ND
Control (i. bodies) *n* = 10	4–51	14·1 ± 9·8	10·1 ± 7·7	9·5 ± 7·9	24·2 ± 17·4	ND
rSmTORed1 *n* = 10	1–31	5·7 ± 4·7	4·7 ± 4·2	2·9 ± 2·3	10·4 ± 8·6	ND
		$65^1/60^2$ %	$62^1/53^2$ %	$67^1/69^2$ %	$64^1/57^2$ %	
		P = 0·0131	*P* = 0·0038	*P* = 0·010	*P* = 0·0058	
Trial 2						
Control (PBS) *n* = 8	12–44	10·3 ± 4·7	11·3 ± 5·9	6·5 ± 4	21·5 ± 10·5	11·75 ± 5·19
rSmTORed1 *n* = 10	5–19	5·5 ± 4·2	6·4 ± 3·2	4 ± 2·5	11·9 ± 5·2	5·88 ± 2·99
		47%,	43%,	38%,	45%,	50%,
		P = 0·0138	*P* = 0·0393	*P* = 0·125	*P* = 0·0212	*P* = 0·0082

Statistical analyses were performed using Student's *t*-test. *P*-values are shown for comparisons between the immunized *versus* both groups of controls taken together (trial 1) and immunized *versus* PBS/CFA injected mice (trial 2). *n*: the number of mice per group (from a total of 10) that were included for analysis. Comparison of numbers of parasites was between animals immunized with rSmTORed1 and the correspondent control groups, PBS-injected animals[1] (comparison 1) and animals injected with inclusion bodies[2] (comparison 2). ND: not determined; i. bodies: inclusion bodies; s.d.: standard deviation.

Detection of SmTOR using sera of immunized BALB/c mice

We wanted to test if antibodies generated in BALB/c mice immunized with rSmTORed1/CFA recognized the antigen on intact *S. mansoni* parasites. When staining cryosections of 3-h schistosomula with anti-rSmTORed1 serum we detected binding of antibodies in anti-rSmTORed1 serum, but not pre-immune serum, to the parasite surface (Fig. 4a). We also observed binding of anti-rSmTORed1 antibodies to 24-h schistosomula when we stained the whole parasite with anti-sera (Fig. 4b).

Furthermore, we tested the binding of sera of BALB/c immunized with irradiated cercariae and found that antibodies recognizing rSmTORed1 were generated even at very low levels compared to levels generated in immunized mice (Supplementary Fig. S7).

Specific anti-rSmTORed1 antibodies are detected in only few *S. mansoni* infected and uninfected humans

Because SmTOR is a surface-exposed receptor [22,33], we tested if *S. mansoni*-infected patients develop specific antibodies to the SmTORed1 domain. Using ELISA, we evaluated the reactivity of anti-rSmTORed1 antibodies in sera of individuals who were infected with *S. mansoni* and uninfected controls. Infection was confirmed by the presence of antibodies against SWA/SEA and by immunofluorescence antibody tests (IFAT). IgG antibodies to rSmTORed1 were detected in only five of 20 patients (25%) infected with *S. mansoni*, but also in four of 40 uninfected individuals (10%) living in Switzerland (Fig. 5a). The positive signal threshold was set arbitrarily considering the relative fold increase values indicated.

To ensure antibody specificity, the five schistosomiasis patients and four uninfected individuals considered to have antibodies against rSmTORed1 (Fig. 5a) were tested in a competition-type ELISA. The specificity of antibody binding against SmTORed1 was evaluated by pre-incubation of diluted patient sera with soluble peptide in the form of Halo-tagged ed1 or HaloTag alone (Supplementary Fig. S1) coupled to magnetic beads via a chloralkane linker [28]. Competition ELISA results show that two out of five schistosomiasis patients (Fig. 5b) and two out of four uninfected individuals (Fig. 5c) have specific anti-rSmTORed1 IgG antibodies whose binding on the peptide could be abolished by pre-incubation of diluted sera with HALOed1, but not with HaloTag alone. This decrease in signal (indicated by an arrow) was significant for the patient sera 4466 and 2006-0059 (Fig. 5b) and normal human sera 42 and 4 (Fig. 5c). These four are the sera in which we had measured the highest positive values for anti-rSmTORed1 antibodies. None of these four sera reacted with C4beta (not shown). All sera from the patients, including the two with anti-rSmTORed1 antibodies, were positive for anti-*S. mansoni* antibodies (anti-SWA and anti-SEA antibodies), whereas this was not the case for the two positive normal sera, indicating that they were not infected by *S. mansoni*.

Discussion

The treatment of *S. mansoni* infection is limited by the availability of only one drug (Praziquantel), and the fact that constant reinfections take place [5,9]. An efficient immune response to antigens that would reduce the infection burden and rate is highly desirable. Here we found SmTOR to be a putative vaccine target in view of the protective effects in mice. Particularly interesting is that this antigen seems not to be recognized in most individuals and mice infected with *S. mansoni*, but was evidently a target for a protective immune response in vaccinated mice. Thus, this antigen might provide a novel approach to reduce infection.

We initially started with the hypothesis that SmTOR might be a good vaccine target, as it is localized in the schistosome tegument membrane. In addition, SmTOR is expressed most highly in *S. mansoni* larvae that are in first contact with the host immune system [22]. Given that its secondary structure contains a relatively large 111 aa surface-exposed extracellular domain, we wanted to see if

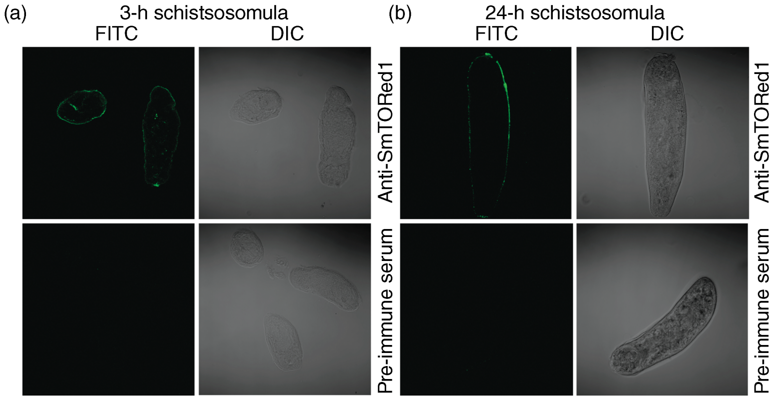

Fig. 4. Sera of immunized mice recognizing *Schistosoma mansoni* tetraspanning orphan receptor (SmTOR) on *S. mansoni* schistosomula. Immunofluorescence [fluorescein isothiocyanate (FITC)] and differential interface images (DIC) of parasite sections (a) or whole parasite (b) visualized by confocal microscopy (×63 magnification). Three-h (a) and 24-h (b) schistosomula labelled with anti-recombinant first extracellular domain of SmTOR (rSmTORed1) serum are shown in the top panels, the respective control sections labelled with pre-immune serum on the bottom panels.

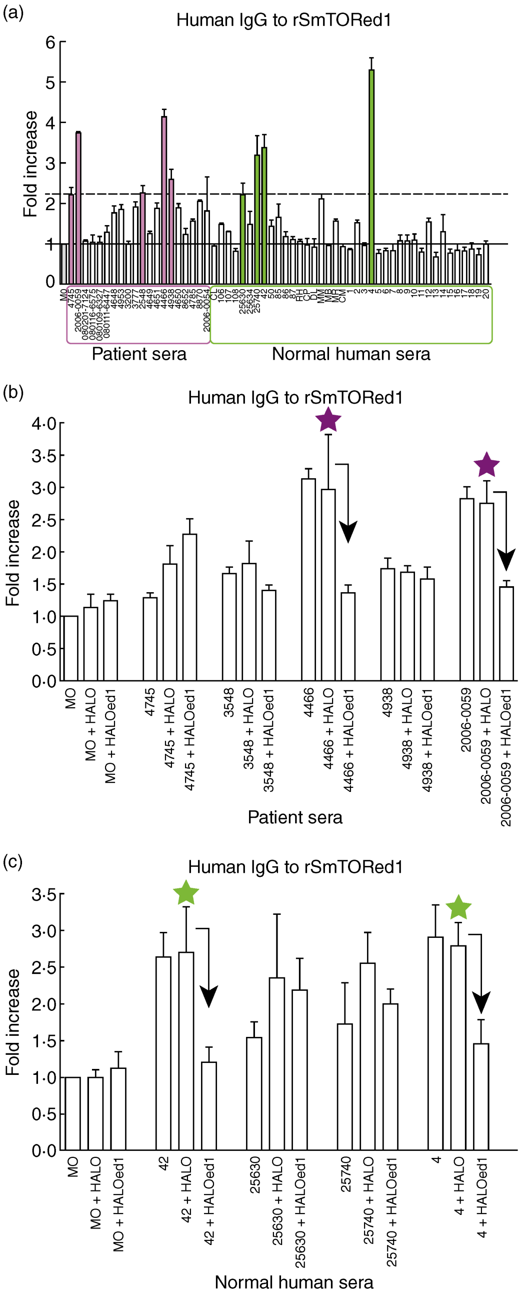

Fig. 5. Detection of human immunoglobulin (Ig)G to recombinant first extracellular domain of *Schistosoma mansoni* tetraspanning orphan receptor (SmTORed1) by enzyme-linked immunosorbent assay (ELISA) and confirmation of specificity by competition ELISA using Halo-tagged bead-bound fusion constructs. (a) Total IgG levels in patients (archived anonymized sera, purple box) and normal human sera (NHS) (green box) normalized to control serum monocytes (MO, grey bar). The black solid line set at 1 corresponds to the mean of absorbance measuring control MO [mean optical density (OD) ± standard deviation (s.d.) ($n = 9$): $\text{OD}_{450\,\text{nm}} = 0{\cdot}176 \pm 0{\cdot}05$], that was set to 1 in order to picture patient and NHS values normalized to one control serum value. The dashed black line was set at an arbitrary threshold and marks the lower limit of a fold-increase value considered as a positive signal. Five (filled purple bars) out of 20 patients and four (filled green bars) out of 40 patients were tested as positive. The results of three different experiments performed in duplicate are shown. Error bars indicate s.d. of the means. (b,c) Measurement of IgG specificity in schistosomiasis patients (b) and NHS (c). OD_{450} values measured were normalized to mean values recorded for the control sample (MO, $\text{OD}_{450\,\text{nm}} = 0{\cdot}243 \pm 0{\cdot}06$, $n = 7$) and indicated as fold increased values. Prior to IgG measurement, individual sera were preincubated with Halo constructs coupled to magnetic beads: no competition (sample + no beads), with HaloTag alone (sample + Halo) or Halo-SmTORed1 (Haloed1). Stars indicate specific IgG to rSmTORed1 in the corresponding serum, tested by competition ELISA. Bars represent the mean values of three independent experiments. Error bars indicate s.d. of the means. Arrows indicate a significant decrease (>40%) in signal due to depletion of specific antibodies by preincubation with Haloed1.

we could find antibodies directed against it in humans, and if this domain would be a target for an efficient immune response in a mouse model.

Hence, we produced SmTORed1 recombinantly in *E. coli* and purified rSmTORed1 by affinity chromatography. We encountered specific difficulties; namely, first, that a highly concentrated SmTORed1 solution led to protein precipitation during denaturant removal, followed by dimerization, as shown on SDS-PAGE. Others have observed this type of reaction for the vaccine candidate Sm14, where protein dimerization and subsequent aggregation led to a reduction in vaccine efficacy [34]. We circumvented this problem by optimizing peptide purification and refolding into 1 × PBS diluting rSmTORed1, which yielded reasonable amounts of soluble protein. As basal expression of rSmTORed1 was observed to have detrimental effects on bacterial viability, bacteria producing protein under the control of the lac repressor [31] were grown in autoinduction media [25], thus circumventing this issue [25,31].

We found that specific humoral and cellular (T cell) responses were generated in BALB/c mice when immunized with rSmTORed1 using CFA/IFA as adjuvant. The infection with *S. mansoni* did not alter the total amount of antibody to rSmTORed1 generated in BALB/c mice and did not alter the cytokine profile elicited by stimulation of the spleen cell cultures with rSmTORed1. Thus, vaccination with rSmTORed1 using CFA/IFA as adjuvant induced a strong immune response in mice that was not altered after infection; however, the infection alone did not elicit any response against SmTORed1, as if this peptide – present on cercaria surface – had not been recognized. Also important is that the same immunization protocol in C57BL/6 mice led to only a minimal antibody response, re-emphasizing that the genetic background is also relevant for the presentation/ processing of the peptide. Interestingly, the two mouse strains used in this study have a known different genetic background, being either prototypical T helper 1 (Th1) (C57BL/6) or Th2 (BALB/c) types [35]. It is known that a schistosome infection, as opposed to immunization, induces Th1 cytokines in C57BL/6 mice at the time of parasite residency in the lung, whereas in BALB/c mice, however, Th2 cytokines are induced with no measurable antibody production [36,37]. Interestingly, exogenous administration of IL-1beta in the days after infection led to the production of specific antibodies against parasite antigens in BALB/c mice associated with a significant reduction in worm burden. By contrast, the same treatment did not induce an antibody response in C57BL/6 mice and even increased the worm burden [36]. An additional example of opposing effects is that immunization with recombinant 3-P dehydrogenase emulsified in CFA, which protected BALB/c but not C57BL/6 mice from infection with *S. mansoni* [38]. Our data point in the same direction.

The vaccination protocol protected the mice significantly against a challenge with *S. mansoni* that were injected subcutaneously in order to obtain a controlled number of cercariae [39]. The worm burden was reduced significantly by 64 and 45% in two independent trials, which meets the criteria defined by the Research and Training in Tropical Diseases/World Health Organization (TDR/WHO) of a 40% reduction of infection [40]. We were able to detect specific IgG to rSmTORed1 in immunized BALB/c mice, and furthermore IgG1 and IgG2a subclasses were both generated to equivalent levels (data not shown). We did not observe a decreasing IgG1/IgG2a ratio, meaning no increase of cytophilic antibodies during the course of the experiment. However, in addition to IgG, we detected specific IgE to rSmTORed1 in response to immunization. In humans the occurrence of schistosome-specific IgE can be associated with protection. An age-dependent increase in production of IgE correlates with resistance to reinfection [41], and there are specific IgE levels detected in adolescents with high resistance to infection [42]. IgE can activate macrophages leading to killing of schistosomula [43,44] and can be involved in eosinophil-, neutrophil- and basophil-mediated killing of schistosomula [45–47]. Antibodies of both subclasses IgG and IgE might target SmTOR immediately, which is expressed in the infectious larvae/ schistosomula migrating to the blood vessels. That we were able to detect binding of sera from immunized mice to *in-vitro*-transformed schistosomula adds some value to this hypothesis. Coating cercariae with antibody may prolong the dwell time in the skin and favour attack by complement. For SmTOR, a non-cytophilic antibody might, in addition, block the natural function of SmTORed1, which is to bind C2 and inhibit complement [41]. Whether the specific antibodies generated or the T cell response were central for the protective effect was not studied further, but it is likely that both participated efficiently in the immune defence.

Several other examples of vaccine candidates meet the target of at least 40% protection in the mouse model [40]. This threshold of protection had been defined in comparison with the radiation-attenuated (RA) cercariae vaccination model that has been shown to be successful in various animal models and induce protection levels of up to 80% [9,48]. This is a strong argument that the development of a vaccine against schistosomiasis is possible. Other arguments include the existence of so-called putatively resistant individuals and the fact that the development of other anti-helminth vaccines has been successful [9]. Unfortunately, RA cercariae are not applicable to humans, but with the development of more sophisticated adjuvants and the discovery of new vaccine candidates there is a realistic chance to achieve the desired goal of an anti-schistosomiasis vaccination for humans [49]. Recombinant Sm14/FABP, a well-established vaccine candidate member, that had been proposed originally by the WHO [40], is the only candidate that is now in clinical trial (http://clinicaltrials.gov/) [50]. Sm23 did not confer protection as a recombinant protein [51], but only when delivered as a DNA vaccine [52]. Sm23 is a member of the so-called tetraspanin family that is present on eukaryotic cells and is also abundant in the tegument of *S. mansoni* [15]. Braschi *et al.* confirmed the presence of four members of tetraspanins on the adult worm surface by proteomic analysis, including Sm23 [17]; all are considered as new vaccine targets. Two of them, TSP-1 and TSP-2, were tested in CBA mice and were shown to have a protective effect of 52 and 64% [53]. In addition, other membrane proteins have been tested in mice. Sm29 [29], and most recently SmStoLP-2 [54], reduce worm burden by 51 and 32%, respectively, in C57BL/6 mice; both vaccines were formulated with CFA/IFA. Both proteins are not only detectable in the tegument of adult worms, but also in skin-stage and lung-stage schistosomula (Sm29) and in 7-day-old *in-vitro*-transformed schistosomula (SmStoLP-2), thus accessible as immune targets at an early time-point after infection. However, the general feeling is that it is unlikely that there is one magic bullet that can induce high levels of protection akin to the one provoked by immunization with RA cercariae [48,55]. A mixture of different peptides may be necessary to obtain an efficient anti-schistosomal vaccine [56]. In addition, the right adjuvant has to be found as the CFA used in the present study has, as yet, no equivalent for humans.

Very puzzling was the presence of specific antibodies in two normal controls living in Switzerland, who did not have antibodies against other antigens of *S. mansoni* and thus were highly unlikely to have been in contact with *S. mansoni*. A possible explanation might be an exposure to bird schistosomes. *Trichobilharzia* spp. are members of the schistosome genera usually infecting birds. They presumably die in human skin after penetration of the non-specific host, causing an inflammatory skin reaction also known as swimmer's itch [57]. Such an infection induces anti-cercaria antibodies recognizing a broad range of antigens in more than 80% of the infected patients [58]. The prevalence of *Trichobilharizia* infection in healthy bathers in the Lake of Geneva is 27% [59]. Whether bird schistosomes possess a TOR homologue to SmTOR that would evoke an immune reaction and be recognized upon infection has not yet been investigated.

The low number of patients having anti-rSmTORed1 antibodies was of particular interest: *Schistosoma mansoni* infection had induced a series of specific antibodies in all these patients, but not anti-rSmTORed1, i.e. 18 of 20 were negative. It might be worthwhile to mention that mice infected with *S. mansoni* without prior immunization did not develop antibodies against SmTOR, suggesting that SmTOR is not recognized at the time of infection. One possible reason for these findings is that *S. mansoni* cercariae, as opposed to *Trichobilharzia*, do not stay in human skin for a long enough period to render a local immune response possible, but move rapidly into the blood vessels. Similarly, cercariae might be transformed rapidly into schistosomula following subcutaneous application in mice, and hence do not evoke an immune response. The absence of specific antibodies in most patients with *S. mansoni* infection suggests that SmTOR might be a very good target for vaccination, as it induces something that natural infection cannot do and that, at least in one mouse model, is efficient.

In conclusion, we found that, despite all the drawbacks discussed, rSmTORed1 might be a new vaccine target against *S. mansoni* infection. The next steps will include testing its vaccine efficacy in an outbred mouse strain and determine whether using other adjuvants acceptable for human use would be sufficient to produce a protective immune response.

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Disclosure

None of the authors has conflicts of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Production of HaloTag, Halo-tagged C4beta and Halo-tagged ed1 as bacterial fusion proteins used covalently coupled to magnetic beads for competition enzyme-linked immunosorbent assay (ELISA). (a) Schematic representation of HaloTag fusion peptides generated. The HaloTag protein (boxed) is attached N-terminally to the sequences of interest via a 16 aa linker containing a tobacco etch virus (TEV) protease cleavage site. The six C-terminal amino acids of the linker sequence remaining attached to the peptides after cleavage are shown (underlined). HaloTag protein linked to *Schistosoma mansoni* tetraspanning orphan receptor (rSmTORed1) loop is denoted as HALOed1 and HaloTag fused to a 26 aa C4β chain stretch is termed HALOC4beta (human C4β chain peptide S^{206}). Local sequence alignment over the C-terminal 27 aa was performed with ClustalW2 and then edited manually (identical amino acids in bold letters, similar amino acids shaded in grey). (b) Protein samples extracted from BL21 4·5 h after induction with isopropyl β-D-1-thiogalactopyranoside (IPTG). HaloTag (HALO) and Halo-tagged (HALOC4 beta, HALOed1) peptides in crude *Escherichia coli* extracts visualized by Coomassie stain (left) and detected by Western blot probed with monoclonal anti-ed1 antibody AbDy04644·1 (right).

Fig. S2. Antibodies against recombinant first extracellular domain of *Schistosoma mansoni* tetraspanning orphan receptor (rSmTORed1) generated in mice do not recognize the homologous stretch on the complement C4 β chain. Total immunoglobulin (Ig) detection in sera of individual BALB/c mice immunized with rSmTORed1 ($n = 3$; mice 1·1–1·3) and a control mouse injected with phosphate-buffered saline (PBS) only (mouse 3·1). rSmTORed1 = purified histidine (HIS)-tagged SmTORed1; rSmTORed1/C4beta/HALO control tobacco etch virus (TEV) cut = SmTORed1, human C4β chain peptide S^{206} (C4beta) produced as HALO-tagged peptides or HALO-tag alone purified by TEV protease digestion.

Fig. S3. Titration of sera from individual mice before and after immunization analysing anti-recombinant first extracellular domain of *Schistosoma mansoni* tetraspanning orphan receptor (rSmTORed1) immunoglobulin (Ig) (IgG, IgM, IgA) responses. Open and filled symbols denote Ig levels determined at days −7 and 35, respectively. Data points for the three mice ($n = 3$) of the strain indicated immunized with antigen and (a,b) complete Freund's adjuvant/incomplete Freund's adjuvant (CFA/IFA) or (c,d) muramyl dipeptide (MDP) adjuvant are shown in the left panel; values for the control mice ($n = 3$) immunized with adujvant/phosphate-buffered saline (PBS) only are shown in the right panel. The dilution determined for further screening of sera in the different settings is boxed. It was set according to optical density (OD) values at 450 nm still within but near the boarder of the plateau reached at low serum dilutions.

Fig. S4. Total immunoglobulin (Ig) amounts in sera of individual mice immunized with recombinant first extracellular domain of *Schistosoma mansoni* tetraspanning orphan receptor (rSmTORed1) and the control groups injected with inclusion bodies measured in sera pre-immunization and after the first boost. Ig levels in mice immunized with rSmTORed1 (antigen, ag; $n = 10$), inclusion bodies (i. bodies; $n = 5$) or PBS ($n = 5$) in complete Freund's adjuvant/incomplete Freund's adjuvant (CFA/IFA) were monitored at days −7 and 35 of the trial. Sera for the enzyme-linked immunosorbent assay (ELISA) assay were diluted as determined by end-point titrations for the different mouse strain adjuvant combinations that was (a) 1:6400 for C57BL/6 mice injected with antigen in CFA/IFA and (b,c) 1:3200 for BALB/c and C57BL/6 mice immunized with muramyl dipeptide (MDP). Statistical analyses used were: Student's *t*-test to compare titre at days −7 and 35 and one-way analysis of variance (ANOVA) to compare titres at day 35; n.s.: statistically not significant; statistically significant *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$).

Fig. S5. Recombinant first extracellular domain of *Schistosoma mansoni* tetraspanning orphan receptor (rSmTORed1) complete Freund's adjuvant/incomplete Freund's adjuvant (CFA/IFA)-induced antibody response in BALB/c mice after challenge with *Schistosoma mansoni* infection. Immunoglobulin (Ig)M (a) and IgA (b) serology in mouse sera sampled at days −7, 35 and 107 (serum dilution both 1:1600). BALB/c mice were immunized with rSmTORed1 (antigen, ag) or control groups immunized with inclusion bodies (i. bodies) or buffer only [phosphate-buffered saline (PBS)] using complete CFA/IFA as adjuvant. All groups were infected with *S. mansoni* cercariae at day 55. Groups consisted of $n = 10$ animals. Arrows indicate injections at days 0 (prime), 21 (boost 1) and 42 (boost 2).

Fig. S6. The cytokine secretion profile of spleen cell cultures stimulated with antigen of immunized BALB/c mice was maintained after the infection challenge. Measurement of cytokines in the supernatant of spleen cell cultures after stimulation with recombinant first extracellular domain of *Schistosoma mansoni* tetraspanning orphan receptor (rSmTORed1) (a) 14 days after the second boost or (b) after immunization challenge experiment (trial 1) after 8 weeks (day 114) of infection. Number of spleens analysed: (a) $n = 3/2$ (rSmTORed1/PBS), (b) $n = 2/2$ (rSmTORed1/PBS).

The bars represent the mean values ± standard deviation (s.d.) of either three or two (rSmTORed1/PBS) different samples each measured in triplicate. Splenocytes of all mice were confirmed to have the capacity for release of cytokines in response to stimulation with concanavalin A.

Fig. S7. Detection of anti-recombinant first extracellular domain of *Schistosoma mansoni* tetraspanning orphan receptor (rSmTORed1)-specific antibodies in sera from BALB/c mice ($n = 5$) immunized with radiation-attenuated cercariae. Total immunoglobulin (Ig) to rSmTORed1 was measured in pre-immune sera (day −7), after the first (day 35) and second boost (day 63). Results for the same sera diluted 1:3200 and 1:6400 are shown.

Table S1. Primer list used for cloning of Halo-tagged fusion constructs.

Appendix S1. Supplementary materials and methods.

8.3 Curriculum Vitae

Curriculum Vitae

Katrin Jane Ingram-Sieber Date of birth: 08.12.1983

Place of birth: Worms
Nationality: German
Adress: Gleimstrasse 51, 10437 Berlin, Germany

Work Experience

05/2010 – today	**PhD student at Swiss Tropical and Public Health Institute, Basel** Thesis Title: "Accelerating antischistosomal drug discovery: Preclinical studies of antimalarials, synthetic peroxides and praziquantel derivatives" Supervised by: Prof. Dr. Jennifer Keiser Collaborations: N. D. Zelinsky Institute of Organic Chemistry/ University of Zürich/ University of Sydney/ University of Nebraska/ Walter Reed Army Institute Additional activities: Supervision of Master students
05/2009 – 11/2009	**Scientific assistant at UniversitätsSpital Zürich, Clinic for Clinical Pharmacology and Toxicology** 2nd half of Practical Year, Staff of Centre for Pharmacovigilance, pharmaceutical information center for physicians, Director of clinic: Prof. Dr. med. G. Kullak-Ublick
11/2009 - 09/2010	**Scientific author for "Apotheke+Marketing"** writing certificated further education articles: „Rheumatoide Arthrithis, der Wirkstoff Tocilizumab" „Weniger Flush unter Niacin-Kombinationstherapie"
11/2008 – 05/2009	**Pharmaceutical intern at Pinocchio Apotheke; Freiburg** 1st half of Practical Year Familiarization with all areas of the pharmacy operations owner: E. Hoffmann-Lang

Education

12/2009	**License to practice pharmacy** Regional council Stuttgart, Baden-Württemberg (3rd state examination; average grade: 1,0 (A))
09/2004 - 09/2008	**Studies of Pharmacy** at Ruprecht-Karls-Universität Heidelberg (2nd state examination; average grade: 1,2 (A))
10/2007 – 02/2008	**Tutor in "Instrumental Analytics for pharmacy students"** at the Ruprecht-Karls-Universität Heidelberg
06/2007 - 09/2007	**Research internship at Pharmacology department** at School of Pharmacy, London research topic: "Phosphorylation of GST-fusion proteins of $GABA_A$ receptor subunits by PKG"; supervisor: Prof. J. Jovanovic
09/2007	1st **state examination in Pharmacy**, average grade: 2, 0 (B)

02/2007 – 03/2007	**Internship at Universitätsklinikum Heidelberg** at department for Clinical Pharmacy, research topic: „Prioritising the prevention of medication handling errors“ (PMID: 18787975)
08/2006	Internship at **Bayer HealthCare,** Leverkusen department: Pharmaceuticals
1994 - 2003	**Gymnasium Hemsbach** A-levels, average grade 1, 4 (A)
Others	**Introductory Course in Laboratory Animal Science**, Zürich 2010

International Experience

07/2003 – 05/2004	**stay abroad in Australia** Combination of travelling and working (with Working Holiday Visa) 08/2003 – 10/2003 Staff at *NOSH Recruitments, Sydney* 01/2004 - 04/2004 Office Assistant at *Travellers Auto Barn, Sydney* (car rental and sell agency)

Other Skills

Languages	**English**	(fluent)
	French	(good knowledge)
	Swedish	(basic knowledge)
IT	Statsdirect, R, MS Word, Excel, Power Point	

Presentations

04/2013	Research seminar, Swiss Tropical and Public Health Institute, “Investigation of antimalarial compounds for the treatment of schistosomiasis”, Basel, Switzerland
12/2012	Swiss Society of Tropical Medicine and Parasitology (SSTMP), Student Meeting, Bern, Switzerland
11/2012	The American Society of Tropical Medicine and Hygiene (ASTMH) 61st ASTMH Meeting, Atlanta, USA
11/2012	Symposium of the British Society of Parasitology (BSP): Emerging Paradigms in Anti-Infective Drug Design (Poster), London, UK
12/2011	Swiss Society of Tropical Medecine and Parasitology (SSTMP), Student Meeting, Basel, Switzerland
09/2011	BioValley Science Day (Poster), Basel, Switzerland
12/2010	Swiss Society of Tropical Medicine and Parasitology (SSTMP), Student meeting, Spiez, Switzerland

List of Publications

1. Meister I, **Ingram-Sieber K**, Cowan N, Todd M, Robertson MN, Meli C, Patra M, Gasser G, Keiser J.
   Activity of praziquantel enantiomers and main metabolites against Schistosoma mansoni.
   Antimicrob Agents Chemother. 2014 Jun 30.

2. **Ingram-Sieber K**, Cowan N, Panic G, Vargas M, Mansour NR, Bickle QD, Wells TN, Spangenberg T, Keiser J.

Orally active antischistosomal early leads identified from the open access malaria box.
PLoS Negl Trop Dis. 2014 Jan 9;8(1):e2610

3. Patra M, **Ingram K**, Leonidova A, Pierroz V, Ferrari S, Robertson MN, Todd MH, Keiser J, Gasser G.
In vitro metabolic profile and in vivo antischistosomal activity studies of (η(6)-praziquantel)Cr(CO)3 derivatives.
J Med Chem. 2013 Nov 27;56(22):9192-8

4. Knopp S, Becker SL, **Ingram KJ**, Keiser J, Utzinger J.
Diagnosis and treatment of schistosomiasis in children in the era of intensified control.
Expert Rev Anti Infect Ther. 2013 Nov;11(11):1237-58.

5. Patra M, Joshi T, Pierroz V, **Ingram K**, Kaiser M, Ferrari S, Spingler B, Keiser J, Gasser G.
DMSO-mediated ligand dissociation: renaissance for biological activity of N-heterocyclic-[Ru(η6-arene)Cl2] drug candidates.
Chemistry. 2013 Oct 25;19(44):14768-72

6. **Ingram K**, Duthaler U, Vargas M, Ellis W, Keiser J.
Disposition of mefloquine and enpiroline is highly influenced by a chronic Schistosoma mansoni infection.
Antimicrob Agents Chemother. 2013 Sep;57(9):4506-11

7. Panic G, **Ingram K**, Keiser J.
Development of an in vitro drug sensitivity assay based on newly excysted larvae of Echinostoma caproni.
Parasit Vectors. 2013 Aug 13;6:237

8. Tsang AS, **Ingram K**, Keiser J, Hibbert DB, Todd MH.
Enhancing the usefulness of cross dehydrogenative coupling reactions with a removable protecting group.
Org Biomol Chem. 2013 Aug 14;11(30):4921-4

9. Patra M, **Ingram K**, Pierroz V, Ferrari S, Spingler B, Gasser RB, Keiser J, Gasser G.
[(η(6) -Praziquantel)Cr(CO)(3)] Derivatives with Remarkable In Vitro Anti-schistosomal Activity.
Chemistry. 2013 Feb 11;19(7):2232-5

10. de Moraes J, Keiser J, **Ingram K**, Nascimento C, Yamaguchi LF, Bittencourt CR, Bemquerer MP, Leite JR, Kato MJ, Nakano E.
In Vitro Synergistic Interaction Between Amide Piplartine and Antimicrobial Peptide Dermaseptin Against Schistosoma mansoni Schistosomula and Adult Worms.
Curr Med Chem. 2013 Jan 1;20(2):301-9

11. Lochmatter C, Schneider CL, **Ingram K**, Keiser J, Schifferli JA.
Schistosoma mansoni tetraspanning orphan receptor (SmTOR): a new vaccine candidate against schistosomiasis.
Clin Exp Immunol. 2012 Dec;170(3):342-57

12. **Ingram K**, Yaremenko IA, Krylov IB, Hofer L, Terent'ev AO, Keiser J.
Identification of antischistosomal leads by evaluating bridged 1,2,4,5-tetraoxanes, alphaperoxides, and tricyclic monoperoxides.
Med Chem. 2012 Oct 25;55(20):8700-11

13. Patra M, **Ingram K**, Pierroz V, Ferrari S, Spingler B, Keiser J, Gasser G.
Ferrocenyl derivatives of the anthelmintic praziquantel: design, synthesis, and biological evaluation.
J Med Chem. 2012 Oct 25;55(20):8790-8.

14. Marxer M, **Ingram K**, Keiser J.
    Development of an in vitro drug screening assay using Schistosoma haematobium schistosomula.
    Parasit Vectors. 2012 Aug 9;5:165

15. **Ingram K**, Schiaffo CE, Sittiwong W, Benner E, Dussault PH, Keiser J.
    In vitro and in vivo activity of 3-alkoxy-1,2-dioxolanes against Schistosoma mansoni.
    J Antimicrob Chemother. 2012 Aug; 67(8):1979-86

16. **Ingram K**, Ellis W, Keiser J.
    Antischistosomal activities of mefloquine-related arylmethanols.
    Antimicrob Agents Chemother. 2012 Jun;56(6):3207-15

17. Keiser J, **Ingram K**, Vargas M, Chollet J, Wang X, Dong Y, Vennerstrom JL.
    In vivo activity of aryl ozonides against Schistosoma species.
    Antimicrob Agents Chemother. 2012 Feb;56(2):1090-2

18. Keiser J, **Ingram K**, Utzinger J.
    Antiparasitic drugs for paediatrics: systematic review, formulations, pharmacokinetics, safety, efficacy and implications for control.
    Parasitology. 2011 Oct;138(12):1620-32

During my studies and PhD studies I have attended lectures and courses given by

C. Klein, N. Metzler-Nolte, A. Jäschke, M. Wink, S. Wölfl, G. Fricker, G. Reich, U. Müller, T. Bertsche, W.E. Haefali, T. Hoppe-Tichy, H. Seidling, J. Huwyler, R. Brun, C. Burri, J. Keiser, J. Utzinger, M. Tanner, P. Mäser, M. Rottmann, I. Felger, S. Wittlin, H.-P. Beck, P. Odermatt, S. Krähenbühl